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WITNESS my hand this
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PROVISIONAL SPECIFICATION

Invention Title: Methods of Diagnosing Prognosing and Treating Activin Associated Diseases and Conditions

Applicant: MONASH UNIVERSITY

The invention is described in the following statement:

METHODS OF DIAGNOSING PROGNOSING AND TREATING ACTIVIN ASSOCIATED DISEASES AND CONDITIONS

FIELD OF THE INVENTION

The present invention relates to methods for diagnosis and prognosis and treatment of activin associated conditions preferably including the activin subunit βc and dimers formed by the dimerisation of activin subunits β_A, β_B, βc, β_D or β_E, or combinations with βc. The invention also provides methods and compositions for preventing or treating conditions and/or diseases associated with activins and activin dimer formation relating to βc.

BACKGROUND

Activins, are members of the TGF-β superfamily that have diverse roles as potent growth and differentiation factors in many organs and tissues. Activins are homo- or heterodimers of activin β subunits, such as β_A, β_B, βc, β_D or β_E that form activin dimer ligands. The activin family encompasses disulfide-linked dimeric proteins characterized by a conserved cysteine-knot motif. Activin A (β_A-β_A) was originally isolated in ovarian follicular fluid as a stimulator of FSH secretion. However it is now recognised that activins such as activin A (β_A-β_A), activin B (β_B-β_B), and activin AB (β_A-β_B) have a range of biological activities that include mesoderm induction in *Xenopus laevis* embryos, immune suppression, bone growth, nerve cell survival, wound healing, tumourogenesis and tissue differentiation in pancreas, kidney and heart (Luisi et al, 2001. Eur J Endocrinol 145:225-36, McDowell et al, 1999. Semin Cell Dev Biol 10:311-7, de Kretser et al, 1999. J Endocrinol 161:195-8, Hubner et al, 1999. Histol Histopathol 14:295-304). However, neither the activin βc subunit nor activin C have been implicated in any of these biological processes. In addition, no biological activity has been determined for activin βc or activin C (βc-βc). For example, Groome et al, (2001, J. Mol. Cell. Endo. 180: 73-77) refer to the "continuing failure of activins C.. to display a bioactivity", Lau et al, (2000, Mol Cel Biol, 20 (16):6127-37) state that "activin betaC ... are not essential for either embryonic development or liver function" and Chang et al (2001, Mol Cell Endocrinol, Jun

30;180(1-2):39-46) state that activin betaC is " not essential for liver growth, differentiation and regeneration".

Some activin family members appear to be involved in differentiation and control of proliferation. Examples of activin dimer ligands involved in these processes include activin A (β_A-β_A), activin B (β_B-β_B), and heterodimer activin AB (β_A-β_B). More recently, activin β_C subunit, along with activin β_D and β_E subunits, have been identified, which form a different subset of activin β subunits, but no biological function of activin C (β_C-β_C) has been identified.

The activin β_C subunit was cloned from mouse (Lau et al, 1996, Biochim Biophys Acta 1307:145-8) and human liver (Hotten G et al, 1995, Biochem Biophys Res Commun 206:608-13)Activin β_D has been cloned from *Xenopus* and microinjection of β_D cDNA induced mesoderm induction, however no mammalian equivalent has been identified (Oda et al, 1995, Biochem Biophys Res Commun 210:581-8). Activin β_E subunit was cloned from mouse liver (9) and found to be expressed in rat liver and lung (O'Bryan et al, 2000, J Mol Endocrinol 24:409-18). Zhang and others demonstrated differences in β_A and β_C mRNA regulation following rat partial hepatectomy and proposed that activin β_C was a liver chalone (Esquela et al, 1997, Biochem Biophys Res Commun 235:553-6, Zhang et al,1997, Endocr J 44:759-64) However, no biological role for activins C, D or E has been established. Activin β_C -β_C forms the activin C homodimer (Kron et al, 1998, J Virol Methods 72:9-14).

Activin β_C subunit has frequently been referred to in the literature as a "liver specific activin". Fang *et al.* described activin β_C expression in adult mice as "a unique liver-restricted pattern" (Fang J et al, 1997, Biochem Biophys Res Commun. 231(3):655-61) Furthermore, Lau *et al.* stated that activin β_C exhibited a "highly restricted tissue expression pattern" in the mammalian liver (Lau et al, 2000. Mol Cell Biol. 20(16):6127-37) while Schmitt *et al.* found "that the inhibin/activin β_C gene is predominantly expressed in adult mouse liver" and that the expression level in liver was "specific and high" (Schmitt et al, 1996, Genomics 32:358-66) In addition, Chang *et al.* described activin β_C as being

"expressed primarily in the liver in the adult" and having a "highly restricted tissue-specific expression pattern" (Chang et al, 2001, Mol Cell Endocrinol. Jun 30;180(1-2):39-46). Also Kron *et al.* stated that "the β_C subunit is exclusively expressed in liver tissue" (Kron et al, 1998, J Virol Methods 72:9-14)

Activin signal transduction is initiated by ligand binding inducing the formation of a heteromeric receptor complex of type I and II transmembrane serine/threonine kinase receptors. Activin binding to ActRII or IIB, results in recruitment and phosphorylation of type I receptor ActRI, thereby initiating the phosphorylation of downstream signaling proteins, the Smad (Sma- and Mad-related) proteins. Following phosphorylation, Smad2 and 3 (receptor-regulated Smads), form a heteromeric complex with Smad4 (co-Smad) and translocate from the cytoplasm to the nucleus (Lebrun et al, 1999, Mol Endocrinol 13:15-23; Wrana and Attisano, 2000, Cytokine Growth Factor Rev 11:5-13; Pangas et al, 2000, Trends Endocrinol Metab 11:309-314.). Interaction of Smad proteins with either transcription factors or DNA-binding elements regulate appropriate gene expression. For example, in *Xenopus*, the DNA binding transcription factor, forkhead activin signal transducer-1 (FAST-1) binds to the Smad2 and Smad4 complex to activate the activin response element (ARE) on the *Xenopus Mix.2* promoter (Chen et al, 1996 Nature 383:691-6; Chen et al, 1997, Nature 389:85-9). It is not known if activin β_C and β_E subunits transduce a signal through the above activin receptors or if they have their own receptors.

Little is known about the presence, significance or function of activin β_C subunit protein in tissues/organs. In addition, little is known about the formation of activin dimers and the regulation of activin dimer formation. In particular, the regulation of dimerisation of activin subunits β_A, β_B, β_C, β_D or β_E, or combinations thereof. Consequently, there remains a need for providing effective methods and compositions for detecting activin β_C subunit protein and modulating activin dimer formation.

It is often difficult to predict a predisposition for a disease and in some cases particularly where a cell or a tissue of a normal individual appears to be very

similar to a tissue which is diseased or at an early stage of disease the difference is not immediately apparent. Hence, it would be desirable to identify an indicator which can provide early indications of a predisposition for a disease or condition compared to a normal or non-diseased cell or tissue.

Hence, it is an object of the invention to identify an indicator of a disease or condition which can provide an insight on the propensity or predisposition of a disease or condition of an individual.

SUMMARY OF THE INVENTION

In a first aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition, the method including detecting an activin β_C subunit and/or an activin dimer including an activin β_C subunit in a cell or biological sample of a subject. Preferably, the method includes the use of an antibody that recognises an epitope of an activin β_C subunit to detect an activin β_C subunit and/or an activin dimer including an activin β_C subunit in a cell or biological sample of a subject. However, the activin βc may be detected by any means. Applicants have found that activin βc levels and bioactivity in many tissues show changes correlating to onset or establishment of a disease or condition. It serves as an indicator of change in tissues and cell function and characteristics of the cell.

In a preferred aspect, the present invention provides a method of diagnosing and/or prognosing a disease or condition of any one of the following organs (either normal tissue, benign disorders or malignant tumours): adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gall bladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, and small intestine, myometrium, benign uterus, fallopian tube, tonsil, seminal vesicle, spleen, soft tissue and appendix the method including detecting an activin β_C subunit and/or an activin dimer including an activin β_C subunit in a cell or biological sample of the organ.

In a further preferred aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition said method including detecting an activin β_C subunit in a cell or biological sample, the method including the steps of:

(a) contacting a first antibody that recognises an epitope of a first activin β subunit with a cell or biological sample;

(b) allowing the first antibody to bind to a first activin β subunit in the cell or biological sample;

(c) washing the cell or biological sample to substantially remove any unbound material in the sample;

(d) contacting the cell or biological sample with a second antibody that recognises an epitope of a second activin β subunit, wherein the second antibody is tagged with a labelling agent; and

(e) detecting the labelling agent to identify an activin β_C dimer in the biological sample, wherein the first or second antibody recognises an epitope of an activin β_C subunit.

In yet another aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition associated with activin dimer formation in a subject, the method including detecting βc subunit in a cell or biological sample including the steps of:

(a) contacting a first antibody that recognises an epitope of a first activin β subunit with a cell or biological sample from a subject:

(b) allowing the first antibody to bind to a first activin β subunit in the cell or biological sample;

(c) washing the sample to substantially remove any unbound material in the cell or biological sample;

(d) contacting the cell or biological sample with a second antibody that recognises an epitope of a second activin β subunit, wherein the second antibody is tagged with a labelling agent; and

(e) detecting the labelling agent to identify an activin β_C dimer in the cell or biological sample, wherein the first or second antibody recognises an epitope of an activin β_C subunit.

Another aspect of the present invention provides a composition for diagnosing and/or prognosing a disease or condition, wherein the composition includes an antibody that recognises an epitope of an activin β_C subunit, and a suitable diluent, excipient or carrier. Preferably, the antibody is a purified antibody that is capable of recognising monomeric or dimeric forms of activin β_C. More preferably, the antibody recognises an epitope of activin β_C that includes the amino acid sequence VPTARRPLSLLYYDRDSNIVKT-DIPDMVVEAC.

In yet another aspect of the present invention there is provided a kit for diagnosing and/or prognosing a disease or condition, wherein the kit includes a first antibody that recognises an epitope of a first activin β subunit, a second antibody that recognises an epitope of a second activin β subunit, and a labelling agent for tagging the second antibody, wherein the first or second antibody recognises an epitope of an activin β_C subunit.

A further aspect of the invention is a method of treating or preventing a disease or condition, the method including detecting the presence of activin β_C subunit in a cell or biological sample of a subject and controlling levels or bioactivity of activin β_C in the subject.

Preferably, the diseases or conditions treated may be diseases or conditions of organs selected from the group including adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, uterus, fallopian tube tonsil, seminal vesicle, spleen, soft tissue and appendix.

Preferably the conditions are selected from the group including pneumonia, gastrointestinal infection, rheumatoid arthritis, inflammatory disorders, cancer or tumors.

In another aspect of the present invention there is provided a pharmaceutical

composition for treating, preventing or diagnosing and/or prognosing a disease or condition, the composition including an effective amount of activin β_C subunit or an activin β_C inhibitory molecule, and a suitable pharmaceutically acceptable diluent, excipient or carrier.

FIGURES

Figure 1 shows immunolocalisation of activin β_C subunit protein in human adrenal and thyroid gland and following the development of cancer in these organs. Insets show low power view of whole tissue section.

(A) The cortex of the adrenal gland displays an isolated nuclear (arrow) staining pattern for activin β_C subunit protein. Weak positive and strong postive (arrowhead) cytoplasmic staining was also observed in the adrenal medulla. (B) Tissue from a patient with adrenal cortical carcinoma displayed predominantly strong nuclear (arrow) activin β_C subunit immunolocalisation, however cytoplasmic (arrowhead) staining was also observed. (C) The follicles of the thyroid gland display intermittent immunolocalisation for activin β_C subunit protein. Predominantly, epithelial cells of the follicles display no staining (arrow) for the activin β_C subunit, however some epithelial cells have cytoplasmic localisation (arrowhead). (D) In contrast, a patient with thyroid minimally invasive follicular carcinoma displayed strong activin β_C subunit staining in the cytoplasm (arrow). (E) In addition, a patient with papillary carcinoma of the thyroid gland immunolocalised strongly to the nuclei (arrow) and was less intense in the cytoplasm (arrowhead).

Figure 2 shows immunolocalisation of activin β_C subunit protein in normal human digestive tissues (stomach, rectum, colon) and following the development of adenocarcimoma. Insets show low power view of whole tissue section.

(A,B) Activin β_C subunit protein was localised to epithelial cells (arrow) of the human stomach, however the staining pattern was variable. Smooth muscle cells and macrophages displayed variable staining. (C) In a patient with

moderately differentiated stomach adenocarcinoma, a pattern of predominantly cytoplasmic staining (arrow) was observed. (D) In contrast, a patient with poorly differentiated stomach adenocarcinoma displayed strong nuclear (arrow) staining, with less intense cytoplasmic (arrowhead) staining. (E) Similarly, in patients with stomach adenocarcinoma that metastasised to the lymph node, strong nuclear staining (arrow) was observed. (F) The benign colon displays strong activin β_C subunit protein immunolocalisation in some secretory epithelial cells (arrow) and smooth muscle cells. Nuclear staining was observed intermittently (G) Tissue from a patient with adenocarcioma of the colon displayed strong nuclear (arrow) and cytoplasmic (arrowhead) staining. (H) The normal rectum displayed both cytoplasmic and nuclear staining of the surface epithelium (I) Rectal adenocarcinoma displayed both nuclear (arrow) and cytoplasmic (arrowhead) staining however this is not observed in all tumour cells.

Figure 3 shows immunolocalisation of activin β_C subunit protein in normal, urinary bladder, skin, breast, lymph node and following the development of cancer in these tissues. Inserts show low power view of whole tissue section.

(A) The transitional epithelium of the urinary bladder immunolocalises activin β_C subunit protein, both the cytoplasm and some nuclei. Intermittent smooth muscle cells also display positive staining. (B) Urinary bladder poorly differentiated carcinoma strongly immunolocalises the nuclei of these tumour cells, however the cytoplasm also shows positive staining. (C) The skin immunolocalises the activin β_C subunit in the cytoplasm of keratinocytes as well as some nuclei, hair follicles, and blood vessels. (D) In tissue from a patient with skin squamous cell carcinoma, activin β_C subunit protein strongly immunolocalises to the nuclei of the tumour cells, however the cytoplasm is also postive. (E) Normal breast epithelium immunolocalises activin β_C subunit protein. Myoepithelial cells displayed both positive (arrow) and negative staining (arrowhead), however the secretory epithelial cells showed strong cytoplasmic localisation (asterisk). (F) In contrast, patients with breast residual infiltrating duct carcinoma display strong nuclear staining, as well as

cytoplasmic localisation. (G) Breast infiltrating lobular carcinoma tissue also displayed predominantly nuclear localisation associated with weak cytoplasmic staining. (H) Tissue from a patient with breast papillary carcinoma displayed strong nuclear and cytoplasmic staining. (I) The normal lymph node tissue immunolocalised activin β_C subunit protein in the stromal tissue (arrow) surrounding the lymphyocytes. However the lymphocytes themselves were negative for the activin β_C subunit (arrowhead). (J) Tissue from a patient with lymphoma displayed strong nuclear staining, however not all nuclei were positive. Some tumour cells displayed cytoplasmic immunolocalisation.

Figure 4 shows immunolocalisation of activin β_C subunit protein in normal human salivary gland, bone, nasal cavity and following the development of cancer in these tissues. Insets show low power view of whole tissue section.

(A) In the salivary gland, cytoplasmic localisation for activin β_C subunit protein is observed in the ducts (arrow), serous cells (arrowhead), mucous cells (asterisk) and nerves of this organ. (B) In a patient with a Warthin tumour of the parotid gland, cytoplasmic and some nuclei staining is observed in the tumour cells. (C) Tissue from a patient with carcinoma of the submandibular gland immunolocalises activin β_C subunit protein to the cytoplasm and the nuclei of these tumour cells. (D) Tissue from a patient with low grade chondrosarcoma, activin β_C subunit protein displayed focal nuclear localisation of chondrocytes. (E) In contrast, tissue from a patient with bone osteosarcoma shows predominant positive staining in the cytoplasm, however there is also some nuclear staining. (F) Both strong cytoplasmic and nuclear staining is observed in a patient with bone giant cell tumour. (G) Tissue from the normal nasal cavity displays activin β_C subunit immunolocalisation in the epithelium of the nasal mucosa. Specifically in both the basal cells (the proliferative area of the epithelium), and more predominantly localised in the secretory epithelial cells. (H) In tissue from a patient with inverted papilloma of the nasal cavity, cytoplasmic and nuclear localisation was observed in the tumour cells.

Figure 5 shows immunolocalisation of activin β_C subunit protein in normal

human stomach and duodenum and following the development of cancer in these tissues. Insets show low power view of whole tissue section.

Normal stomach tissue immunolocalises activin β_C subunit protein in both the glands and smooth muscle, however this localisation is intermittent with both positive and negative staining. (A) In normal tissue, glands displayed both nuclear and cytoplasmic immunolocalisation but staining was non-uniform. (B) In the antrum of the stomach displays immunolocalisation in both the mucosa and muscle layers, but not all cells are positive. For example, the gastric surface displays cytoplasmic localisation. (C) The duodenum immunolocalises activin β_C subunit protein in both the mucosal and smooth muscle cell layer. Not all cell types are positive and localisation is non-uniform. In the luminal surface secretory cells, some cells that display activin β_C subunit localisation in the cytoplasm, while others have nuclear staining in the deeper layers of the mucosa. (D) Tissue from a patient with moderately differentiated stomach adenocarcinoma displayed predominantly cytoplasmic activin β_C subunit immunolocalisation. (E) In contrast, both nuclear and cytoplasmic immunolocalisation was observed in a patient with poorly differentiated stomach adenocarcinoma. (F) Nuclear staining was also observed in a patient with signet ring cell carcinoma of the stomach, in addition to stromal staining. (G) Tissue from lymphoma of the stomach displayed a similar pattern of staining in the nuclei of tumour cells and stromal cells. (H) Stomach carcinoma that had metastasised to the lymph node, displayed intermittent nuclear, cytoplasmic and stromal localisation.

Figure 6 shows immunolocalisation of activin β_C subunit protein in normal human gallbladder and urinary bladder and following the development of cancer in these tissues. Insets show low power view of whole tissue section.

(A) In the normal gallbladder, basal and secretory cells localise the activin β_C subunit. Both nuclear and cytoplasmic staining is observed in the epithelial cell layer. Smooth muscle localisation was also observed. (B) Similarly, tissue from a patient with adenocarcinoma of the gallbladder displayed both nuclear and

cytoplasmic staining in the tumour cells. In addition, smooth muscle (asterisk; inset) in the vicinity of the tumour cells displayed strong activin β_C subunit protein localisation. (C) In tissue from the urinary bladder, the transitional epithelium immunolocalises activin β_C subunit protein, in a predominantly a cytoplasmic pattern, however some cells do display nuclear immunolocalisation. (D) Tissue from a patient with high grade transitional cell carcinoma of the urinary bladder, immunolocalises activin β_C subunit protein in a both cytoplasmic and nuclear pattern in these tumour cells. (E) In addition, poorly differentiated carcinoma cells have strong cytoplasmic and strong nuclear staining.

Figure 7 shows immunolocalisation of activin β_C subunit protein in normal adrenal gland and uterine cervix and following the development of cancer in these tissues. Insets show low power view of whole tissue sections.

(A) In the cortex of the adrenal gland, activin β_C subunit protein is observed in the cytoplasm, however this localisation is variable with both weak and strong areas of staining. In addition, nuclear localisation in occasionally observed. (B) Tissue from a patient with cortical carcinoma of the adrenal gland displays strong cytoplasmic and nuclear staining. (C) The uterine cervix displays some nuclear staining, however not all cells are positive. Both the cytoplasm and nuclei immunolocalise the activin β_C subunit in squamous dysplasia. (D) Tissue from a patient with squamous cell carcinoma of the uterine cervix immunolocalises the activin β_C subunit protein in the cytoplasm (arrowhead) of tumour cells. Some tumour cells also display prominent nuclear (arrow) localisation.

Figure 8 shows immunolocalisation of activin β_C subunit protein in the normal pancreas and esophagus and following the development of cancer in these tissues. Insets show low power view of whole tissue sections.

(A) The pancreas immunolocalises activin β_C subunit protein strongly in the secretory granules of the acinar cells (arrowhead) and more weakly to the islet

cells (arrow). (B) Tissue from a patient with pancreatic cancer displayed stronger activin β_C subunit localisation in the tumour cells. Both cytoplasmic and nuclear staining was observed in the tumour cells. (C) In the esophagus, activin β_C subunit immunolocalisation was observed in blood vessels and some smooth muscle. However, apart from some sporadic nuclear positive cells, the epithelial layer was negative. (D) Tissue from a patient with squamous cell carcinoma, strongly localised activin β_C subunit protein in the cytoplasm of the tumour cells.

Figure 9 shows immunolocalisation of activin β_C subunit protein in normal human thyroid and thymus and following the development of cancer in these tissues. Insets show low power view of whole tissue sections.

(E) In the normal thyroid gland, activin β_C subunit protein localisation in the epithelial cells of the thyroid follicles is intermittent and the gland is predominantly negative. The positive cells may have both cytoplamsic and nuclear staining. (F) In contrast, tissue from a patient with minimally invasive follicular carcinoma of the thyroid displayed strong localisation in the cytoplasm of the tumour cells. (G) In the normal thymus, lymphocytes are negative for the activin β_C subunit (arrowhead), however the thymic epithelium (arrow) displays cytoplasmic and weak nuclear staining. Stromal cells (asterisk) are also positive. (H) In tissue from a patient with thymoma, the tumor cells display strong activin β_C subunit protein cytoplasmic localisation. The lymphocytes remain negative for activin β_C subunit protein with malignancy.

Figure 10 shows immunolocalisation of activin β_C subunit protein in human myometrium, benign uterus and fallopian tube. Insets show low power view of whole tissue sections.

(A) In the myometrium, activin β_C subunit protein immunolocalisation is weak or negative. (B) Tissue from a patient with leiomyoma of the uterus displayed positive staining in smooth muscle cells. Some nuclear staining was also observed. (C) The fallopian tube immunolocalised activin β_C subunit protein in secretory cells, some intermittent nuclear staining was also present.

Figure 11 shows immunolocalisation of activin β_C subunit protein in normal human tonsil, seminal vesicle, spleen, and appendix. Insets show low power view of whole tissue sections.

(A) In the tonsil, activin β_C subunit protein localised to the stromal cells (arrow) but not the lymphocytes (arrowhead). (B) In the spleen, blood vessels are strongly positive (arrow), while the lymphoid aggregations (arrowhead) are negative. (C) The secretory epithelial cells of the seminal vesicle displayed both cytoplasmic (arrowhead) and nuclear (arrow) staining for the activin β_C subunit. Smooth muscle cells were also positive. (D) The cytoplasm of the secretory epithelial cells (arrowhead) in the appendix strongly immunolocalise activin β_C subunit protein, however some nuclear staining (arrow) is also observed.

Figure 12 shows immunolocalisation of activin β_C subunit protein in the normal and diseased human brain. Insets show low power view of whole tissue sections.

(A) In tissue from a patient with glioblastoma, the benign region displays astroctyes that strongly immunolocalise activin β_C subunit protein in the cytoplasm (arrow). Reactive astrocytes are also positive. (B) In the same patient, the blood brain barrier (arrow) also strongly localises the activin β_C subunit. (C) The cytoplasm of glioblastoma tumour cells (arrow) are positive for activin β_C subunit protein. (D) Tissue from a patient with meningioma also strongly localises activin β_C subunit protein in the cytoplasm of the tumour cells. (E) The grey matter of the human brain displays positive staining in neuronal cells. Activin β_C subunit protein immunolocalises to the white matter (F), the cerebellum (G) and the pituitary gland (H) of the human brain.

Figure 13 shows immunolocalisation of activin β_C subunit protein in the normal brain of the sheep and both wild type and transgenic mice that express a human Cu,Zn Superoxide Dismutase mutation resulting in neruodegenerative disease.

Both the transgenic mice brain (A) and wild type (B) mouse brain display activin β_C subunit localisation in cerebellum. The molecular layer strongly displays activin β_C subunit protein (arrow), the granular layer displays less staining (asterisk) and the Purkinje cells (arrowhead) are negative. (C) The endocrine cells (arrow) of the sheep pituitary gland immunolocalise activin β_C subunit protein. (D) In the pre-optic area of the sheep brain, neuronal cells with axon processes (arrow) localise the activin β_C subunit. (E) In the sheep hypothalamus neuronal cells (arrow) display activin β_C subunit protein localisation.

Figure 14 shows immunolocalisation of activin β_C subunit protein in malignant human skin, larynx, tongue, small intestine and disorders of the appendix and soft tissue.

(A) Tissue from a patient with melanoma displays activin β_C subunit localisation in the cytoplasm and nuclei of tumour cells. (B) In a patient with pseudomyxoma of the appendix, cytoplasmic and some nuclear staining is observed. (C) Activin β_C subunit protein immunolocalises to the cytoplasm and some nuclei in a patient with neurofibromatosis of the soft tissue. (D) Tissue from a patient with squamous cell carcinoma of the larynx displays cytoplasmic and some nuclear staining. (E) Similarly, squamous cell carcinoma of the tongue immunolocalises activin β_C subunit protein in the cytoplasm with some focal nuclear staining. (F) Tissue from a patient with malignant stromal tumour of the small intestine displayed strong activin β_C subunit protein localisation. (G) In the normal small intestine, non-uniform activin β_C subunit localisation was observed in the epithelial cells.

DESCRIPTION OF THE INVENTION

In a first aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition, the method including detecting an activin β_C subunit and/or an activin dimer or bioactivity of a β_C subunit including an activin β_C subunit in a cell or biological sample of a subject. Preferably, the method includes the use of an antibody that recognises an epitope of an activin β_C

subunit to detect an activin β_C subunit and/or an activin dimer including an activin β_C subunit in a cell or biological sample of a subject. However, the activin β_C may be detected by any means. Applicants have found that β_C levels and bioactivity in many tissues show changes correlating to onset or establishment of a disease or condition. It serves as an indicator of change in tissues and cell function and characteristics of the cell. Accordingly, the invention serves to identify tissues and cells having a propensity or predisposition for a disease or a condition. Preferably, the presence of β_C provides an indication or a predisposition or propensity for a cell or biological tissue to become cancerous or tumorigenic. This therefore provides a diagnostic for a subject for any disease or condition that is preferably associated with β_C.

Preferably, the disease or condition is associated with activin β_C.

The term "activin β_C" as used herein includes full length activin β_C subunit protein, an active portion thereof, or an activin β_C subunit variant that is capable of dimerising with another activin subunit, such as activin β_A, β_B, β_C, β_D or β_E. Preferably, the activin β_C is capable of dimerising with activin β_A subunit to form activin heterodimer AC. An activin β_C variant may include activin β_C which has been modified at the nucleotide or amino acid level and may include additions or deletions or replacements of nucleotides or amino acids which do not affect the functionality of the protein. Activin β_C may be natural or recombinant and therefore may be induced to be expressed in a cell or biological sample. The activin β_C may be from any animal species, preferably the activin β_C is encoded by mammalian DNA, more preferably the activin β_C is human, mouse or rat activin β_C.

An activin dimer may include a homodimer or heterodimer formed by activin subunits selected from the group consisting of β_A, β_B, β_C, β_D or β_E. Preferably, the activin dimer including an activin β_C subunit detected is selected from the group consisting of activin AC (β_A-β_C), activin BC (β_B-β_C), activin C (β_C-β_C),

activin CD (β_C-β_D) or activin CE (β_C-β_E). Most preferably, the activin β_C dimer to be detected is activin AC (β_A-β_C). An activin dimer present in a cell or biological sample can be detected by general methods of assaying for the specific activin dimer forms. Such assays preferably utilise an antibody that recognises an epitope of an activin β_C subunit. Suitable assays for detecting activin dimer formation may preferably include ELISA, immunohistochemistry, immunoprecipitation, immunoaffinity purification or Western Blot techniques.

Activin β_C has a structure similar to other activins and other members of the TGFβ superfamily. The structure of activins are based on the conservation of the number and spacing of the cysteines within each subunit and the disulphide linkages between the two subunits that form characteristic cysteine knots. Other similarities relate to dimer formation, the location of the bioactive peptide in the carboxy terminal region of the precursor activin subunit molecule and similar intracellular signalling mechanisms. Human activin β_C, in comparison with other TGF-β superfamily members, reveals a typical structure with 9 conserved cysteines and a large precursor molecule that contain a core of hydrophobic amino acids at the N terminus thought to be the secretion signal sequence (Hotten G et al, 1995, Biochem Biophys Res Commun 206:608-13). The mouse activin β_C also contains 9 conserved cysteines, and N terminal hydrophobic amino acids that may serve as a signal peptide Schmitt et al, 1996, Genomics 32:358-66).

Activin β_C may be obtained from methods of producing monomeric and dimeric activin β_C in CHO cells (Biopharm GmbH, Heidelberg, Germany), bacterial cells or mammalian cells. Activin β_C monomer and dimer can also be obtained from methods involving insect larvae infected with recombinant baculovirus (Kron et al, 1998, J Virol Methods 72:9-14)

In the specification the term "cell(s)" is taken to include any cells. Preferably, the cells are derived from a mammalian species, such as, but not limited to, human, mouse, bovine, sheep or other domestic animals. It is preferred that the cells are selected from the group including, but not limited to normal, cancer

or tumour cells of the fibroblast, epidermal, adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, benign uterus, fallopian tube, tonsil, seminal vesicle, spleen, soft tissue and appendix. The cells may be normal cells, diseased cells, adult cells or embryonic cells.

The cells may be single cells, cultured cells or part of a tissue. The cells may be genetically modified recombinant cells, such as transgenic cells. Preferably, the cells express activin β_C. The cells may be part of a whole animal thereby providing an *in vivo* diagnosis of the disease or condition in a cell. The cells may also be derived from a cell line. Preferably, the cells are derived from cell lines derived from, but not limited to adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, benign uterus, fallopian tube, tonsil, seminal vesicle, spleen, soft tissue and appendix.

In the specification the term "biological sample" is taken to include, but not be limited to, serum, tissue extracts, body fluids, cell culture medium, extracellular medium, supernatants, biopsy specimens or resected tissue. The biological sample may include cells as described earlier. Preferably, the biological sample is derived from a mammalian organism, most preferably a human subject. More preferably, the biological sample is selected from the group including, but not limited to, serum, tissue culture supernatant, seminal plasma, cell lysates, tissue homogenates, biological fluids, cerebrospinal fluid and seminal fluid.

Applicants have surprisingly found activin βc subunit protein in human tissues of the following organs (both normal and malignant tumours): adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, and small

intestine.

Activin βc subunit protein was also detected by applicants in human tissues of the following organs (both normal or disorders of): myometrium, benign uterus, fallopian tube, tonsil, seminal vesicle, spleen, soft tissue and appendix.

These results are surprising as the activin βc subunit has frequently been referred to in the literature as a "liver specific activin". Fang *et al.* described activin βc expression in adult mice as "a unique liver-restricted pattern" (Fang J et al, 1997, Biochem Biophys Res Commun. 231(3):655-61) Furthermore, Lau *et al.* stated that activin βc exhibited a "highly restricted tissue expression pattern" in the mammalian liver (Lau et al, 2000. Mol Cell Biol. 20(16):6127-37) while Schmitt *et al.* found "that the inhibin/activin βc gene is predominantly expressed in adult mouse liver" and that the expression level in liver was "specific and high" (Schmitt et al, 1996, Genomics 32:358-66) In addition, Chang *et al.* described activin βc as being "expressed primarily in the liver in the adult" and having a "highly restricted tissue-specific expression pattern" (Chang et al, 2001, Mol Cell Endocrinol. Jun 30;180(1-2):39-46). Also Kron *et al.* stated that "the βc subunit is exclusively expressed in liver tissue" (Kron et al, 1998, J Virol Methods 72:9-14)

In a preferred aspect, the present invention provides a method of diagnosing and/or prognosing a disease or condition of any one of the following organs (both normal and malignant tumours): adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, and small intestine, myometrium, benign uterus, fallopian tube tonsil, seminal vesicle, spleen, soft tissue and appendix the method including detecting an activin βc subunit and/or an activin dimer or bioactivity of a βc subunit including an activin βc subunit in a cell or biological sample of the organ.

Applicants have shown that changes in βc subunit follow development of a

disease or condition such as cancer. The change in βc levels or bioactivity provide an indicator and correlates with the change in the tissue of the organ.

The term "antibody" as used herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they bind specifically to a target antigen. Antibodies may be obtained from commercial sources.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method, isolated from phage antibody libraries, or may be made by recombinant DNA methods. The monoclonal antibodies may also be obtained from commercial sources.

Therefore, suitable antibodies specific to activin β_C can include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and a Fab expression library. For preparation of monoclonal antibodies directed towards activin β_C protein, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. Such techniques include, but are not restricted to, the hybridoma technique originally developed by Kohler and Milstein (Kohler G, Milstein C 1975, Nature 256:495-7)

, the trioma technique, the human B-cell hybridoma technique (Kozbar 1983 Immunology Today 4:72), and the EBV hybridoma technique to produce human monoclonal antibodies (Cole 1985 Monoclonal Antibodies and Cancer Therapy, In. Alan R. Liss, Inc: 77-96).

Various procedures known in the art may be used for the production of polyclonal antibodies to an activin β_C protein. For production of the antibody, various host animals can be immunized by injection with activin β_C protein, such host animals include, but are not limited to, rabbits, mice, rats, etc. Various adjuvants can be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freud's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as bacillus Calmette-Guerin (BCG) and corynebacterium parvum.

Suitable antibodies that specifically bind to activin β_C can be introduced into a cell in numerous fashions, including, for example, microinjection of antibodies into a cell (Morgan and Roth, 1988, Immunol Today 9:84-8) or transforming hybridome mRNA encoding a desired antibody into a cell (Burke and Warren, 1984, Cell 36:847-56).

Antibody fragments can be generated by techniques known in the art. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulphide bridges of the $F(ab')_2$ fragment, the Fab fragments that can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In a further technique, recombinant antibodies specific to activin β_C protein can be engineered and ectopically expressed in a wide variety of cell types to bind to activin β_C as well as to block activin β_C from dimerising.

The preparation and use of antibodies according to the present invention may be achieved using techniques well known in the art, and include various antibody labeling techniques and applications. Suitable labels for antibodies include, but are not limited to, radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. The antibody may also be treated prior to adding the label, for example by biotinylation.

The term "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label itself may be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. Labelling may include the addition of a subsequent step with a label for example, biotin step, then strepavidin-alkaline phosphatase label.

Labelling of the antibody may be achieved directly or indirectly. Well known conjugation methods may be used for attaching labels to antibodies. Preferably, after labelling, unbound label is removed from the labelled antibody using purification procedures known to those of skill in the art. The antibody may also be fractionated to provide an immunoglobulin fraction such as IgG or IgM fractions. These antibody fractions may be isolated using methods known to those in the art including using recombinant protein G for IgG or immunoprecipitation for IgM.

It is most preferred that the activin βc or dimers including βc are detected by an antibody, wherein the antibody recognises an epitope of an activin β_C subunit. Preferably, the antibody is capable of recognising monomeric or dimeric forms of activin β_C. More preferably, the antibody recognises an epitope of activin β_C that includes the amino acid sequence VPTARRPLSLLYYDRDSNIVKTDIPDMVVEAC or an equivalent thereof. It is preferred that the antibody is a monoclonal antibody. Preferably, the antibody is

specific to an activin β_C subunit. More preferably, the antibody is specific to the human activin β_C subunit. The antibody may be a mouse monoclonal antibody developed against the human activin β_C subunit. Most preferably, the antibody does not cross react with activin β_A, β_B or β_E peptides.

The activin β_C antibody may be used in a number of methods and diagnostic techniques. For instance, the activin β_C antibody may be used in ELISA, immunohistochemistry, immunoaffinity purification, immunoprecipitation, Western Blot and antibody binding studies to detect the activin β_C. Preferably, the activin β_C antibody may be used in ELISA methods for diagnostic purposes, such as diagnosing and/or prognosing activin related diseases. Human and/or animal serum, tissues, fluids, culture supernatants may be used in assays based on activin β_C antibody. The activin β_C antibody of the present invention may also be used as an inhibitory molecule to inhibit activin β_C activity and binding.

In a further preferred aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition said method including detecting an activin β_C subunit in a cell or biological sample, the method including the steps of:

(f) contacting a first antibody that recognises an epitope of a first activin β subunit with a cell or biological sample;

(g) allowing the first antibody to bind to a first activin β subunit in the cell or biological sample;

(h) washing the cell or biological sample to substantially remove any unbound material in the sample;

(i) contacting the cell or biological sample with a second antibody that recognises an epitope of a second activin β subunit, wherein the second antibody is tagged with a labelling agent; and

(j) detecting the labelling agent to identify an activin β_C dimer in the biological sample, wherein the first or second antibody recognises an epitope of an activin β_C subunit.

Preferably, the disease or condition is associated with activin βc subunit.

Preferably, the activin β_C dimer detected is selected from the group consisting of activin AC (β_A-β_C), activin BC (β_B-β_C), activin C (β_C-β_C), activin CD (β_C-β_D) or activin CE (β_C-β_E). Most preferably, the activin β_C dimer to be detected is activin AC (β_A-β_C). In the method it is preferred that the first antibody recognises an epitope of an activin β_C subunit. Preferably, the second antibody recognises an epitope of an activin β_A or β_B subunit. More preferably, the second antibody recognises an epitope of an activin β_A subunit. Preferably, step (e) includes quantifying the amount of an activin β_C dimer in the biological sample.

The biological sample used in the method may include samples as previously discussed. Such samples, may preferably include serum, tissue culture supernatant, seminal plasma, cell lysates, tissue homogenates, biological fluids, cerebrospinal fluid, seminal fluid. Preferably, the biological sample is from a mammalian animal. More preferably, the biological sample is from a human.

In step (a) of the method a first antibody that recognises an epitope of a first activin subunit is contacted with a biological sample. Preferably, the first antibody is coated on a plate, such as a 96 well plate and the biological sample is added to the coated plate. The biological sample may be added neat or in a diluted form. The first antibody coated on a plate is typically referred to as the "capture antibody". In the present method it is preferred that the first antibody recognises an epitope of an activin β subunit. Most preferably, the first antibody is a purified antibody is that is capable of recognising monomeric or dimeric forms of activin β_C. The antibody preferably recognises an epitope of activin β_C that includes the amino acid sequence VPTARRPLSLLYYDRDSNIV-KTDIPDMVVEAC. Preferably, the antibody is specific to activin β_C and more preferably is a monoclonal antibody. The first antibody may be a highly specific activin β_C mouse, anti-human or anti-rat antibody.

The biological sample may be pretreated before contacting the sample with the first antibody. For instance, the sample may be diluted with a suitable diluent, such tissue culture media and/or PBS. The sample may preferably be

denatured with SDS by heating before contacting the sample with the first antibody. The biological sample may preferably be treated to oxidise the sample. More preferably, activin β_C subunit in the sample is oxidised, such that a methionine on an activin β_C subunit is oxidised. A suitable oxidising agent, such as H_2O_2 may be added to the biological sample to oxidise the methionine on an activin β_A subunit.

In a preferred embodiment, the method includes the additional step of adding a dissociating agent to the sample to remove binding proteins. Preferably, the dissociating agent is added before step (a). Preferably, the binding protein removed is selected from the group consisting of follistatins, BMPs or α-2 macroglobulins. SDS may preferably be added to sample as a dissociating agent to remove binding proteins such as follistatins, BMPs, α-2 macroglobulins and others). However other dissociating agents include those published in McFarlane et al, 1996 (Eur J Endocrinol 134:481-9) which describes sodium deoxycholate, Tween 20, SDS as useful dissociating agents. Binding proteins such as follistatin bind to the β subunits of activin A, B with high affinity, and inhibin A and B with lower affinity. Follistatin may also bind to the activin β_C subunit. Therefore, it is preferable to include the dissociating step to remove binding proteins.

In step (b) of the method the first antibody is allowed to bind to a first activin β subunit in the sample. This is preferably achieved by incubating the first antibody and the biological sample under suitable conditions. For instance, suitable media including BSA and/or PBS may be used, preferably activin free serum is used. Most preferably, the sample is incubated over night in a humidifed environment.

In step (c) of the method the sample is washed to substantially remove any unbound material in the sample. The sample is washed in any suitable washing solution, preferably including water or PBS. The sample is preferably washed such that the labelled antibody specifically binds to the target activin subunit.

In step (d) of the method the sample is contacted with a second antibody that recognises an epitope of a second activin β subunit. Preferably, the second antibody recognises an epitope of an activin β_A, β_B, β_C, β_D or β_E subunit. More preferably, the second antibody recognises an epitope of an activin β_A subunit. The second antibody may be a monoclonal or polyclonal antibody and may be generated by methods previously discussed.

The second antibody is required to be tagged with a labelling agent. The preparation and use of antibodies according to the present invention may be achieved using techniques well known in the art, and include various antibody labeling techniques and applications. Suitable labels for antibodies include, but are not limited to, radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. The antibody may also be treated prior to adding the label, for example by biotinylation.

The second antibody that is tagged by a labelling agent as hereinbefore described is typically referred to as the "tag antibody" and is preferably used in a colour detection method. The second antibody may be bound to a labelling agent, such as biotin wherein detection of the label is measured by a coloured enzyme reaction product. Other labelling preferably includes using activin β subunit antibody directly labelled with alkaline phosphatase.

In step (e) of the method an activin dimer that is bound to the second labelled antibody is detected. The method of detection would depend on the labelling agent used to tag the second antibody and then addition of strepavidin alkaline phosphatase. The detection preferably involves colour detection from kit reagents. For instance, colour may be read using a microplate reader using a standard. Calculations on levels or bioactivity of activin AC are based on a standard curve of known amounts of activin AC. For instance, bovine follicular fluid and a human recombinant or purified activin AC protein may be used as a standard for the activin AC assay. Preferably, step (e) includes quantifying the amount of an activin β_C dimer in the biological sample.

In an alternative embodiment, the method may be performed in the reverse way (swapping the capture and tag antibodies). For example, an activin β_A antibody may be coated on the plate and an activin β_C antibody may be labelled. However, this is less preferable due to the high amounts of activin A (β_A-β_A) in certain samples which would cause decreased sensitivity of the assay.

In a further aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition, the method including detecting levels or bioactivity of activin β_C subunit and/or activin β_C dimer formation in a cell or biological sample of a subject. Preferably, the activin β_C dimer formation detected is activin AC (β_A-β_C), activin BC (β_B-β_C), activin C (β_C-β_C), activin CD (β_C-β_D) or activin CE (β_C-β_E). Most preferably, the activin β_C dimer formation detected is activin AC (β_A-β_C).

Preferably, the disease or condition is associated with βc subunit.

The level of activin βc may be measured by any method which indicates a level of activin βc such as, but not limited to, absolute concentrations from a standard curve, relative to a control sample or immunohistochemically with an antibody reactive to the activin βc subunit. For instance, if a tissue is believed to be potentially cancerous, the level of βc subunit can be measured against normal tissue. Differences in activin βc subunit may indicate to type of subunit formed in the cell or biological tissue. Similarly, just differences in the levels or bioactivity of activin βc in the cell can indicate abnormal tissue.

Bioactivity of the βc subunit may be determined by the ability of the βc subunit to induce or decrease activin dimer formation. The dimer formation may be measured by the presence of the β subunit by using an antibody to the subunit.

In the methods of the present invention, the disease or condition associated with activin βc or activin dimers or dimer formation may include diseases or conditions of the adrenal gland, thyroid gland, stomach, colon, rectum, urinary

bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, benign uterus, fallopian tube tonsil, seminal vesicle, spleen, soft tissue and appendix.

In particular, the disease or condition may include inflammatory conditions (eg rheumatoid arthritis, pneumonia, gastrointestinal infection). Preferably the disease is cancer or a tumour.

Applicants have detected activin β_C protein in normal and / or tumours of the following organs: adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, benign uterus, fallopian tube tonsil, seminal vesicle, spleen, soft tissue and appendix. The presence of βc and the difference in patterns of the normal and tumor tissues indicates a change in cells and tissue characteristics indicative of disease or changes in the condition of the cells or tissue.

The present application demonstrates the localization of β_C subunits to specific cell types. The immunohistochemical localization of activin β_C subunits in specific cell types show that activin β_C subunit monomer and its homo- or heterodimers may be formed in these cells.

In yet another aspect of the invention there is provided a method of diagnosing and/or prognosing a disease or condition associated with activin dimer formation in a subject, the method including detecting βc subunit in a cell or biological sample including the steps of:(a) contacting a first antibody that recognises an epitope of a first activin β subunit with a cell or biological sample from a subject:

(b) allowing the first antibody to bind to a first activin β subunit in the cell or biological sample;

(c) washing the sample to substantially remove any unbound material in the

cell or biological sample;

(d) contacting the cell or biological sample with a second antibody that recognises an epitope of a second activin β subunit, wherein the second antibody is tagged with a labelling agent; and

(e) detecting the labelling agent to identify an activin β_C dimer in the cell or biological sample, wherein the first or second antibody recognises an epitope of an activin β_C subunit.

Preferably, the activin β_C dimer detected is selected from the group consisting of activin AC (β_A-β_C), activin BC (β_B-β_C), activin C (β_C-β_C), activin CD (β_C-β_D) or activin CE (β_C-β_E). Most preferably, the activin β_C dimer to be detected is activin AC(β_A-β_C). In the method it is preferred that the first antibody recognises an epitope of an activin β_C subunit. Preferably, the second antibody recognises an epitope of an activin β_A or β_B subunit. More preferably, the second antibody recognises an epitope of an activin β_A subunit. Preferably, step (e) includes quantifying the amount of an activin β_C dimer in the cell or biological sample. The steps of the method may be performed as previously described for detecting an activin β_C subunit.

In the diagnostic methods of the present invention it is preferred that the subject is a mammalian animal, including but not limited to a human. The biological sample of the subject is preferably serum, tissue culture supernatant, seminal plasma, cell lysates, tissue homogenates, biological fluids, cerebrospinal fluid, or seminal fluid. The biological sample may be a lysate of tissue or conditioned media of cells, particularly if the disease or condition to be diagnosed is cellular. If the disease or condition to be diagnosed is related to a reproductive disease or condition then the biological sample may include ovarian follicular fluid, seminal fluid or seminal plasma.

Applicants have detected activin AC protein in samples of human serum from patients with pneumonia and gastrointestinal infection, supernatant from cultured cells from a patient with rheumatoid arthritis, serum from sheep with acute inflammatory condition

Another aspect of the present invention provides a composition for diagnosing and/or prognosing a disease or condition, wherein the composition includes an antibody that recognises an epitope of an activin β_C subunit, and a suitable diluent, excipient or carrier. Preferably, the antibody is a purified antibody is that is capable of recognising monomeric or dimeric forms of activin β_C. More preferably, the antibody recognises an epitope of activin β_C that includes the amino acid sequence VPTARRPLSLLYYDRDSNIVKT-DIPDMVVEAC.

Preferably the disease or condition is associated with activin βc.

The compositions as herein before described preferably include a suitable diluent, excipient or carrier that is compatible with the antibody that recognises an epitope of an activin β_C subunit. An acceptable carrier, excipient or diluent may include, water, salt solutions, BSA, Triton X-100. Preferably, the compositions are sterile aqueous solutions. The compositions may also contain buffers, diluents and other suitable additives. The compositions may include other adjunct components that are compatible with the antibody that recognises an epitope of an activin β_C subunit, such as labelling agents or dyes.

In yet another aspect of the present invention there is provided a kit for diagnosing and/or prognosing a disease or condition, wherein the kit includes a first antibody that recognises an epitope of a first activin β subunit, a second antibody that recognises an epitope of a second activin β subunit, and a labelling agent for tagging the second antibody, wherein the first or second antibody recognises an epitope of an activin β_C subunit.

Preferably the disease or condition is associated with activin βc.

In the kits of the present invention the first antibody and the second antibody may be antibodies as previously described for the methods of the present invention. The first antibody preferably recognises an epitope of an activin β_C subunit. Preferably, the second antibody recognises an epitope of an activin β_A,

β_B, β_C, β_D or β_E subunit. More preferably, the second antibody recognises an epitope of an activin β_A subunit. Preferably, the first or second antibody is a purified antibody is that is capable of recognising monomeric or dimeric forms of activin β_C. More preferably, the antibody recognises an epitope of activin β_C that includes the amino acid sequence VPTARRPLSLLYYDRDSNIVKT-DIPDMVVEAC.

A further aspect of the invention is a method of treating or preventing a disease or condition in a subject, the method including detecting the presence of activin β_C subunit in a cell or biological sample of a subject and controlling levels or bioactivity of activin β_C in the subject.

Without being limited by theory, a subject may be treated to increase or decrease levels or bioactivity of activin β_C. Levels or bioactivity of activin β_C may be preferably increased in a cell and/or biological fluid of a subject by introducing regulatory factors that increase the expression of activin β_C into a cell, introducing expression vectors that express activin β_C into a cell and/or introducing exogenous activin β_C into a cell and/or biological fluid of a subject.

Levels or bioactivity of activin β_C can be increased or decreased by suppressing or enhancing expression of activin β_C. Suitable antisense oligonucleotide sequences (single stranded DNA fragments) of activin β_C may also be used to decrease the levels or bioactivity of activin β_C. These may be created or identified by their ability to suppress the expression of activin β_C. The production of antisense oligonucleotides for a given protein is described in, for example, Stein and Cohen, 1988 (Cancer Res 48:2659-68) and van der Krol et al., 1988 (Biotechniques 6:958-976).

It is preferred that the method of treating or preventing a disease or condition associated with activin βc includes decreasing levels or bioactivity of active activin β_C by the use of an inhibitory molecule. Preferably, the activin β_C inhibitory molecule is an antibody against activin β_C, an activin β_C antisense oligonucleotide or an agent that decreases the expression of activin β_C.

Preferably, the activin β_C inhibitory molecule is an antibody. Suitable antisense oligonucleotide sequences (single stranded DNA fragments) of activin β_C may also be used to decrease the levels or bioactivity of activin β_C. In the method, the activin β_C inhibitory molecule can be preferably administered to a subject. More preferably, the inhibitory molecule is administered in a safe and effective amount into a cell and/or biological fluid of a subject.

The method can include administering to a subject in need thereof an effective amount of an agent that decreases the expression of activin β_C such that the activin dimer formation is induced. Preferably, the agent is an activin β_C inhibitory molecule such as an agonist or antagonist of activin βc such as an antibody.

The term "effective amount" means a dosage sufficient to provide treatment or prevention for the disease or condition being treated or prevented. This will vary depending on the subject and the disease/condition being effected. The effective amounts of an agent used in the methods of the present invention may vary depending upon the manner of administration, the condition of the animal to be treated, and ultimately will be decided by the attending scientist, physician or veterinarian.

The agent, activin β_C inhibitory molecule, activin β_C regulatory factor and/or activin β_C used in the methods as hereinbefore described can be administered systemically or locally to a subject. Systemic administration can be achieved parenterally (e.g. intravenous injection, intramuscular, subcutaneous or intraperitoneal injection, or by implantation of a sustained release formulation), orally, by inhalation, or transdermally (e.g. iontophoretic patch). Local administration to an animal can be achieved by subcutaneous injection, implantation of a sustained release formulation, or transdermal administration. Preferably, the agent, inhibitory molecule, regulatory factors and/or activin β_C is administered directly to prostate tissue of a subject. Topical administration in the form of ointments, aqueous compositions including solutions and

suspensions, liposomes, micro capsules, creams, lotions, aerosol sprays or dusting powders may be used.

In the present methods of treatment activin β_C subunit expression may be increased or decreased by preferably affecting activin β_C expression intracellularly, so to either increase or reduce available activin β_C subunit for heterodimerisation. Therefore, preferably the agent is inserted into a viral vector, such as gene therapy agent that is tissue specific.

Preferably, the diseases or conditions treated may be diseases or conditions of organs selected from the group including adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas, esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, uterus, fallopian tube tonsil, seminal vesicle, spleen, soft tissue and appendix.

Preferably the diseases or conditions are selected from the group including pneumonia, gastrointestinal infection, rheumatoid arthritis, inflammatory disorders, cancer or tumors.

In another aspect of the present invention there is provided a pharmaceutical composition for treating, preventing or diagnosing and/or prognosing a disease or condition, the composition including an effective amount of activin or an activin β_C inhibitory molecule, and a suitable pharmaceutically acceptable diluent, excipient or carrier. Preferably, the pharmaceutical composition includes an activin β_C inhibitory molecule and is suitable for treating.

Preferably, the disease or condition is associated with activin βc subunit.

Preferably, the diseases or conditions treated may be diseases or conditions of organs selected from the group including adrenal gland, thyroid gland, stomach, colon, rectum, urinary bladder, skin, breast, lymph node, human salivary gland, bone, nasal cavity, duodenum, gallbladder, uterine cervix, pancreas,

esophagus, thyroid, thymus, brain, larynx, tongue, small intestine, myometrium, uterus, fallopian tube tonsil, seminal vesicle, spleen, soft tissue and appendix.

Preferably the conditions are selected from the group including pneumonia, gastrointestinal infection, rheumatoid arthritis, inflammatory disorders, cancer or tumors.

The activin β_C inhibitory molecule in the composition may be any be any molecule capable of blocking the activity and/ or expression of activin β_C. Activin β_C inhibitory molecules may include an antibody against activin β_C, an activin β_C antisense oligonucleotide or an agent that decreases the expression of activin β_C.

Preferably, the activin β_C inhibitory molecule suitable for the compositions of the present invention is a purified antibody, wherein the antibody recognizes an epitope of an activin β_C subunit. Preferably, the antibody is capable of recognizing monomeric or dimeric forms of activin β_C. More preferably, the antibody recognizes a epitope of activin β_C that includes the amino acid sequence VPTARRPLSLLYYDRDSNIVKTDIPDMVVEAC. Alternatively, the activin β_C inhibitory molecule is an activin β_C antisense oligonucleotide or an agent that decreases the expression of activin β_C.

The compositions of the present invention can be formulated as pharmaceutical compositions. The compositions may be formulated as solutions, emulsions, or liposome-containing formulations. The compositions may be generated from a variety of components that include liquids, self-emulsifying solids and self-emulsifying semisolids. The pharmaceutical emulsions may also be present as multiple emulsions that are comprised of more than two phases. Pharmaceutical excipients such as emulsifiers, surfactants, stabilisers, dyes, penetration enhancers and anti-oxidants may also be present in the compositions.

Suitable pharmaceutically acceptable carriers can include, water, salt solutions,

alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium sterate, silicic acid and viscous paraffin. Formulations for topical administration may include sterile and non-sterile aqueous solutions. The compositions can also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension and may also contain stabilisers. The solutions may also contain buffers, diluents and other suitable additives. The compositions can include other adjunct components that are pharmaceutically compatible with the active components, such as dyes, flavouring/aromatic agents, preservatives, antioxidants, thickening agents.

The compositions can be conveniently presented in unit dosage form and can be prepared according to conventional techniques in the pharmaceutical field. The compositions can be prepared by combining the active compounds/agents with a liquid carrier or finely divided solid carriers or both. The pharmaceutical compositions may be formulated into many forms, such as, tablets, capsules, liquid syrups, soft gels, suppositories or enemas.

The pharmaceutical compositions of the present invention may be formulated and used as foams, including emulsions, microemulsions, creams, jellies and liposomes. The formulations of the above compositions described would be known to those skilled in the pharmaceutical field.

The methods as hereinbefore described may be performed *in vitro* or *in vivo* and are applicable to various animal species that express activin β_C.

The present invention will now be more fully described with reference to the accompanying Figures and examples that illustrate preferred embodiments of the invention. It should be understood, however, that the description following is a non-limiting example only and should not be taken in any way as a restriction on the generality of the invention hereinbefore described.

EXAMPLES

Example 1: Detection of Activin AC heterodimers in Biological Samples.

(a) Activin AC enzyme linked immunosorbent assay (ELISA)

Plates were coated and blocked as previously described (Evans et al, 1998,. J Endocrinol156:275-82) with human activin β_C subunit Clone 1 monoclonal antibody on 96-well ELISA plates (MaxiSorp; Nunc, Roskilde, Denmark). bFF was used as an interim standard. The top dose in the assay, equivalent to a 1/10 dilution, was assigned the arbitrary unitage of 10 U/ml. Standards and samples were diluted in DMEM / 5% FCS, as used in the culture experiments. 125 µl of a 6% sodium dodecyl sulphate (SDS) solution in PBS was added (3% final concentration, w/v) to 125 µl of sample or standard, mixed, boiled for 3 minutes and allowed to cool. The addition of PBS to the SDS solution was found to improve the performance of the assay and the linearity of the dose-response curve of the standard and samples. Thereafter, 20 µl of 30% H_2O_2 (2% final concentration, v/v) was added and the tubes incubated at room temperature for 30 mins. To each well, was added 25 µl of 20% BSA / 0.1 M Tris / 0.9% NaCl/ 5% Triton X-100 /0.1% sodium azide prior to the addition of 100µl duplicates of the treated samples. Plates were incubated overnight in a sealed humidified box. The next day, the plates were washed with 0.05M Tris / 0.9% NaCl / 0.05% Tween-20 / 0.1% NaN_3 before 50 µl biotinylated E4 monoclonal antibody directed to the activin β_A subunit in 5%BSA / 0.1M Tris / 0.9% NaCl/ 5% Triton X-100 /0.1% sodium azide was added to each well and incubated for 2 hours at room temperature. After washing, alkaline phosphatase linked to streptavidin (Invitrogen Corporation, Carlsbad, CA) was added to the wells and incubated at room temperature for one hour. After further washes, the alkaline phosphatase activity was detected using an amplification kit (ELISA Amplification System; Invitrogen) whereby the substrate was incubated for one hour at room temperature, followed by the addition of an amplifying reagent. The reaction was stopped with the addition of 50 µl of 0.4M

H_2SO_4. The plates were read at 492nm with a 630nm reference filter on a Multiskan RC plate reader (Labsystems, Helsinki, Finland) and data were processed using Genesis Lite EIA software (Labsystems).

(b) Activin AC heterodimer formation, *in vivo*

Activin AC protein levels (U/ml) were measured in samples of human and sheep serum and human cell line supernatants (see Table 1 below).

Changes in activin AC levels (compared to control serum) were observed in serum from patients with pneumonia and gastrointestinal infection.

Activin AC protein could be measured in human cell lines including a rheumatoid arthritis cell line.

Activin AC protein was detected in serum from a sheep with acute inflammation.

Table 1

Sample	Activin AC (U/ml)
Normal serum	
Male serum control	0.033
Female serum control	0.038
Inflammation	
Pneumonia serum	0.094
Gastrointestinal Infection serum	0.090
Sheep acute Inflammation model	0.080
Arthritis	
Rheumatoid arthritis cell line	0.183
Control media	0.168

<u>Example 2: Activin β_C subunit protein immunohistochemistry in normal/diseased human tissues and animal tissues</u>

(a) Human tissues

Human normal tissue array (AA) and human tumor tissue array (BB) were obtained from SuperBioChips Laboratories (Seoul, Korea).

(b) Animal tissues

The left sagittal brain was removed from a transgenic mouse with a neurodegenerative disorder (familial amyotrophic lateral sclerosis) and corresponding wild type animals (38). The tissue was fixed in 4% paraformaldehyde, processed to paraffin and 3 μm tissue sections were cut.

Ewes were killed by i.v. injection of 20ml of Lethobarb (Virbac, Peakhurst, NSW, Australia). The heads were then perfused with 21 ml of heparinized saline followed by 11 ml of 10% formalin fixative solution and 0.51 ml of the same fixative solution containing 20% sucrose. The brain blocks were left overnight in the same fixative containing 30% of sucrose and then in 30% sucrose in PBS until they sank. The brain blocks were then frozen in dry ice, wrapped parafilm and stored at –20°C until sectioning. Coronal sections (7μm) of sheep pituitary were cut on a cryostat, thaw mounted onto superfrost slides and stored at -20° until used. Coronal sections of sheep brain (40 μm) were cut on a cryostat, collected into individual tissue culture wells containing cryoprotectant and stored at -20°C until used.

After being de-paraffinated the tissue underwent a pretreatment step of microwave heating in 0.1M glycine (pH 4.5). The sections were immunostained for activin β_C subunit protein using the DAKO Autostainer (DAKO, Carpinteria, USA). Briefly, endogenous peroxidase was blocked by incubation of sections with 0.03% H_2O_2 for 5 minutes (DAKO, Carpinteria, USA). After incubation with CAS Blocking solution (Zymed, CA, USA) for 10 minutes, the sections were incubated with activin β_C antibody (working concentration 0.45 μg/ml) for 60 minutes. The antibody was detected by incubation with Envision polymer-anti-mouse-horse radish peroxidase (DAKO, Carpinteria, USA) for 15 minutes and visualised by reaction with diaminobenzidine (DAB) (DAKO, Carpinteria, USA) for 5 minutes. The specificity of immunostaining was examined by pre-incubation of primary antibody with 100-fold (w/w) excess of corresponding activin β_C subunit peptide.

(c) Immunolocalisation of activin β_C subunit protein in normal and diseased human tissues.

The activin β_C subunit protein was demonstrated to immunolocalise to most benign and malignant human organs studied. Both cytoplasmic and / or nuclear staining was commonly observed and changes in these patterns occurred between the benign and malignant state. Figures 1 - 14 fully describe the staining pattern and the descriptions below indicate some of the significant findings.

The adrenal gland and thyroid gland, as shown in Figure 1, displayed strong activin β_C subunit protein localisation in malignancy. Staining in the adrenal and thyroid glands shows increased intensity in a malignant state.

Most of the adenocarcinomas of the stomach, colon and rectum (Figure 2) showed a pattern of both cytoplasmic and nuclear activin β_C subunit localisation. This staining pattern differed to the variable and predominantly cytoplasmic staining observed in the normal stomach, colon, and rectum.

Strong activin β_C subunit protein nuclear staining became apparent in the development of malignancy in the skin, breast and lymph node (Figure 3). Some nuclear staining was observed in some cells of the benign skin and breast, however stronger staining was displayed in malignant tissue. Similarly, cytoplasmic localisation of activin β_C subunit protein was observed in the normal salivary gland and nasal cavity however this staining showed strong nuclear localisation, as well as cytoplasmic, in malignancy (Figure 4). In addition, little staining was observed in chondrosarcoma (a benign condition of the bone), however strong nuclear and cytoplasmic activin β_C subunit protein localisation was observed in malignancy. The normal stomach (Figure 2 and 5) displayed variable cytoplasmic localisation. In addition to the stomach adenocarcinomas described above, other stomach malignancies displayed nuclear localisation (and stromal localisation) including stomach signet ring cell carcinoma, stomach lymphoma and metastatic stomach carcinoma. The normal bladder has little

nuclear staining however following the development of cancer, strong nuclear staining was observed (Figure 6).

Some organs, such as the gallbladder, adrenal, uterine cervix, and pancreas had varying degrees of nuclear and cytoplasmic staining in the benign and malignant state (Figure 6, 7, 8).

The esophagus, thyroid and thymus showed little or no staining in the normal tissue, however following the development of cancer increased activin β_C subunit protein localisation was observed (Figure 8, 9).

Other tissues that immunolocalised activin β_C subunit protein included the myometrium, fallopian tube, tonsil, spleen, appendix and seminal vesicle as well as benign disorders of the uterus (Figure 10 and 11).

Normal, damaged and malignant skin immunolocalised different patterns of activin β_C subunit protein staining. Both nuclear and cytoplasmic staining was observed in the normal skin and tumours including squamous cell and melanoma (Figure 3 and 14).

The normal breast immunolocalised the activin β_C subunit and different breast tumours (residual infiltrating duct carcinoma, breast infiltrating lobular carcinoma, breast papillary carcinoma) also displayed cytoplasmic or nuclear localisation (Figure 3).

The brain displays strong activin β_C subunit protein localisation in both the benign and malignant disorders. In particular, astrocytes, blood brain barrier and neurons strongly localise activin β_C subunit (Figure 12). The endocrine cells of the sheep and human pituitary and the neuronal cells of the cerebellum, pre-optic area and hypothalamus display activin β_C subunit localization (Figure 13). Strong localisation is also observed in tumour cells of the brain, in particular tumour cells in (I) glioblastoma of two patients and (II) meningioma of four patients (Figure 12).

The discussion of prior art documents, acts, devices and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in the Australia before the filing date of this application.

Finally, the invention as hereinbefore described is susceptible to variations, modifications and/or additions other than those specifically described and it is to be understood that the invention includes all such variations, modifications and/or additions which fall within the scope of the description as hereinbefore described.

Figure 1

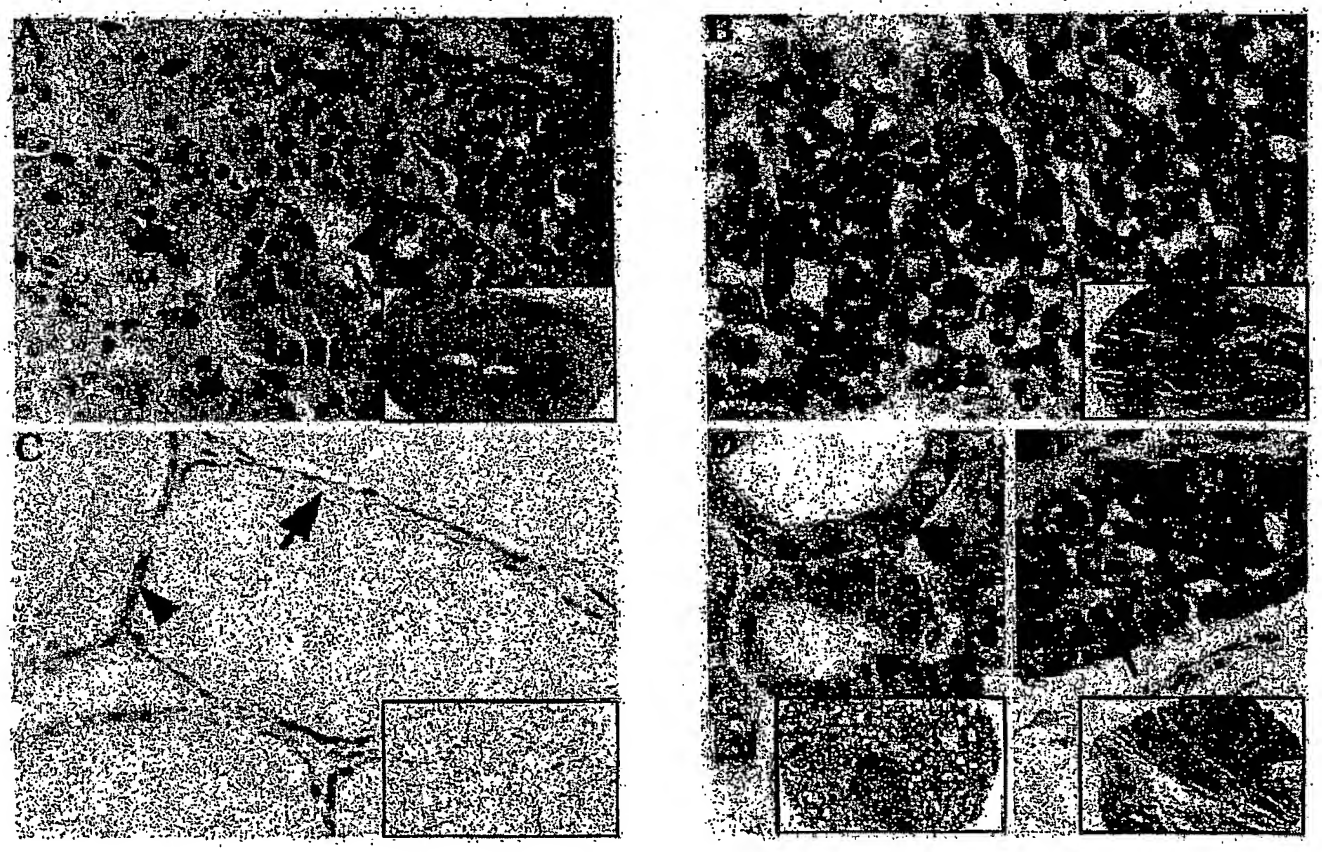

Figure 2

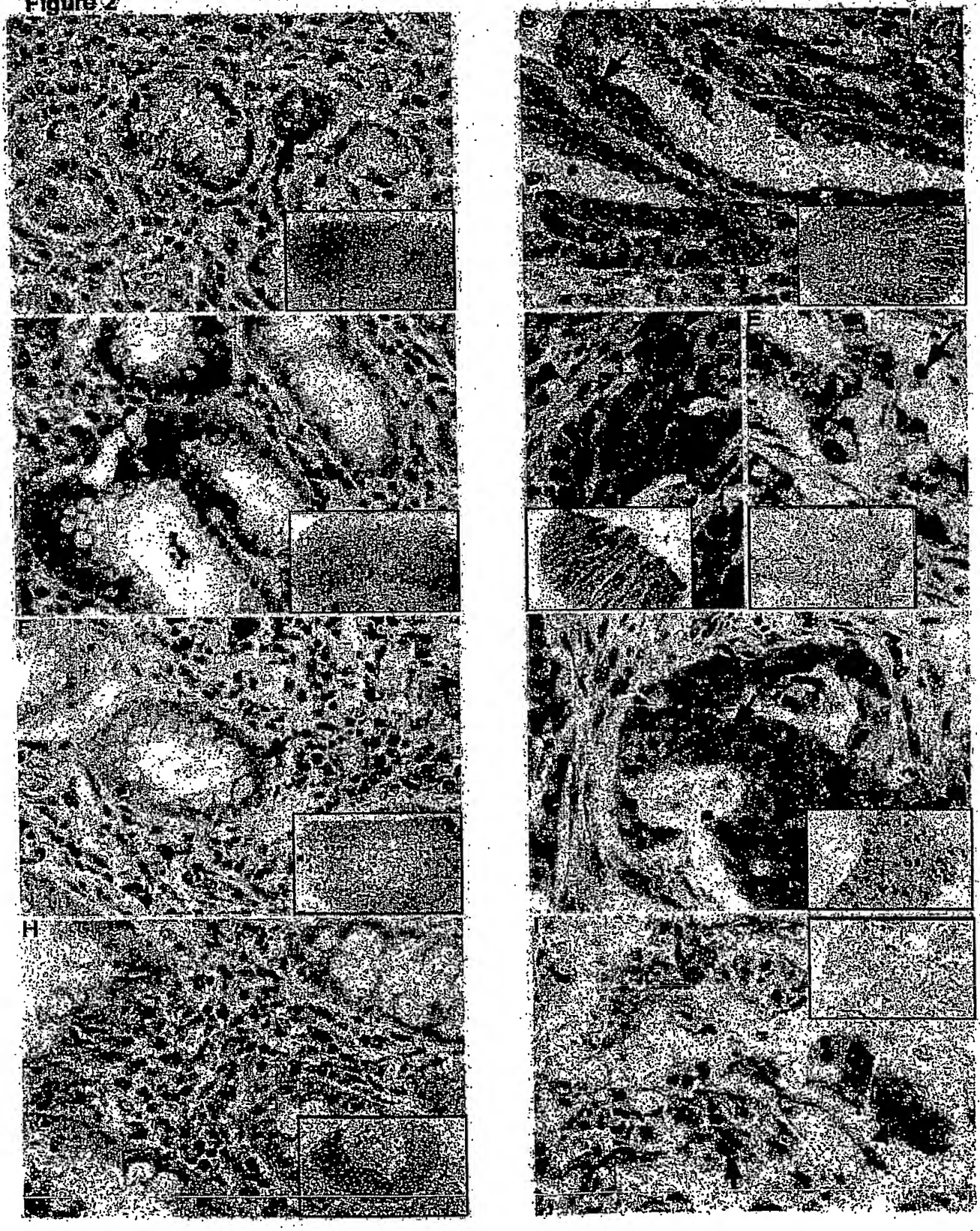

Figure 3

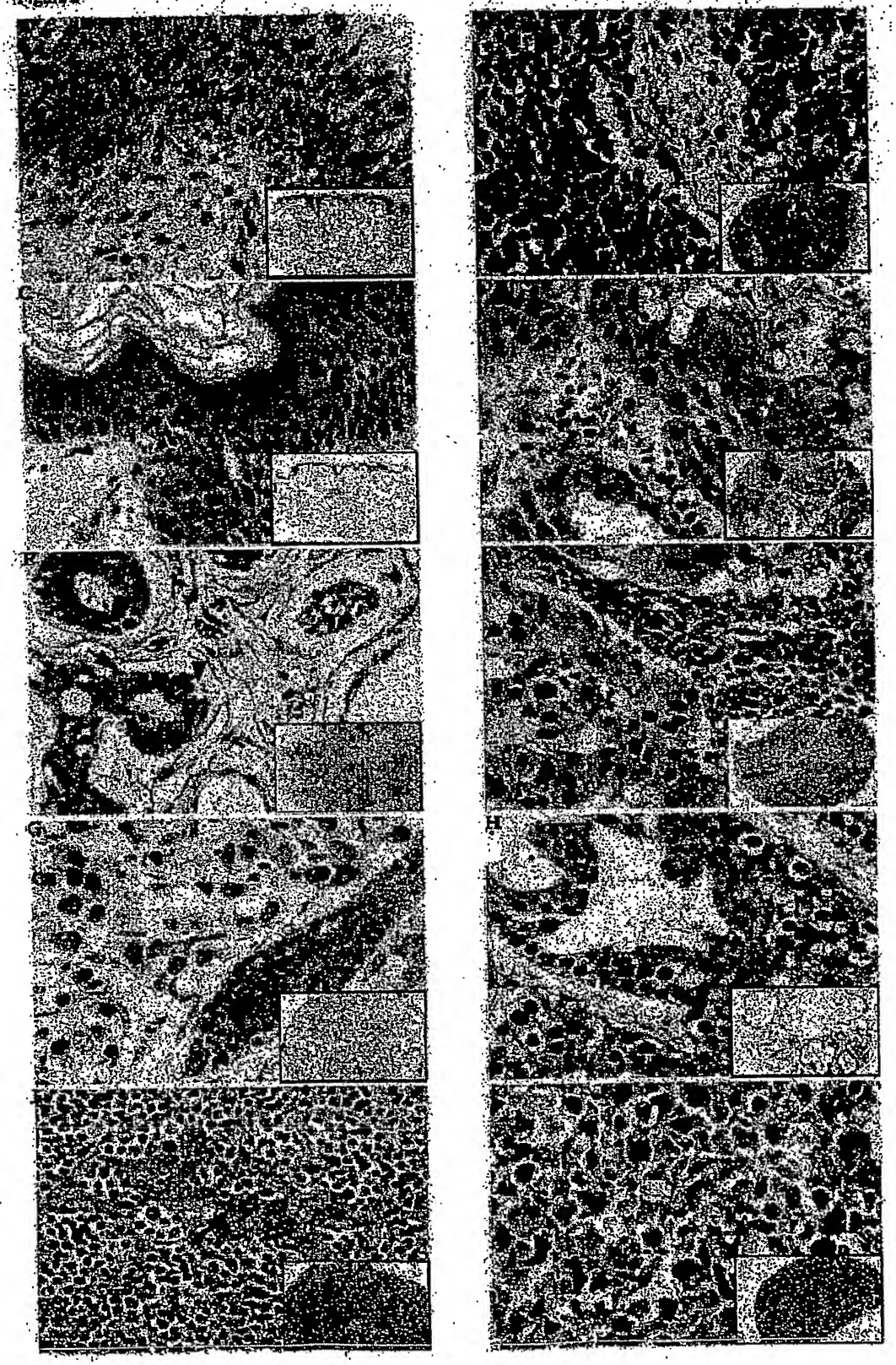

Figure 4

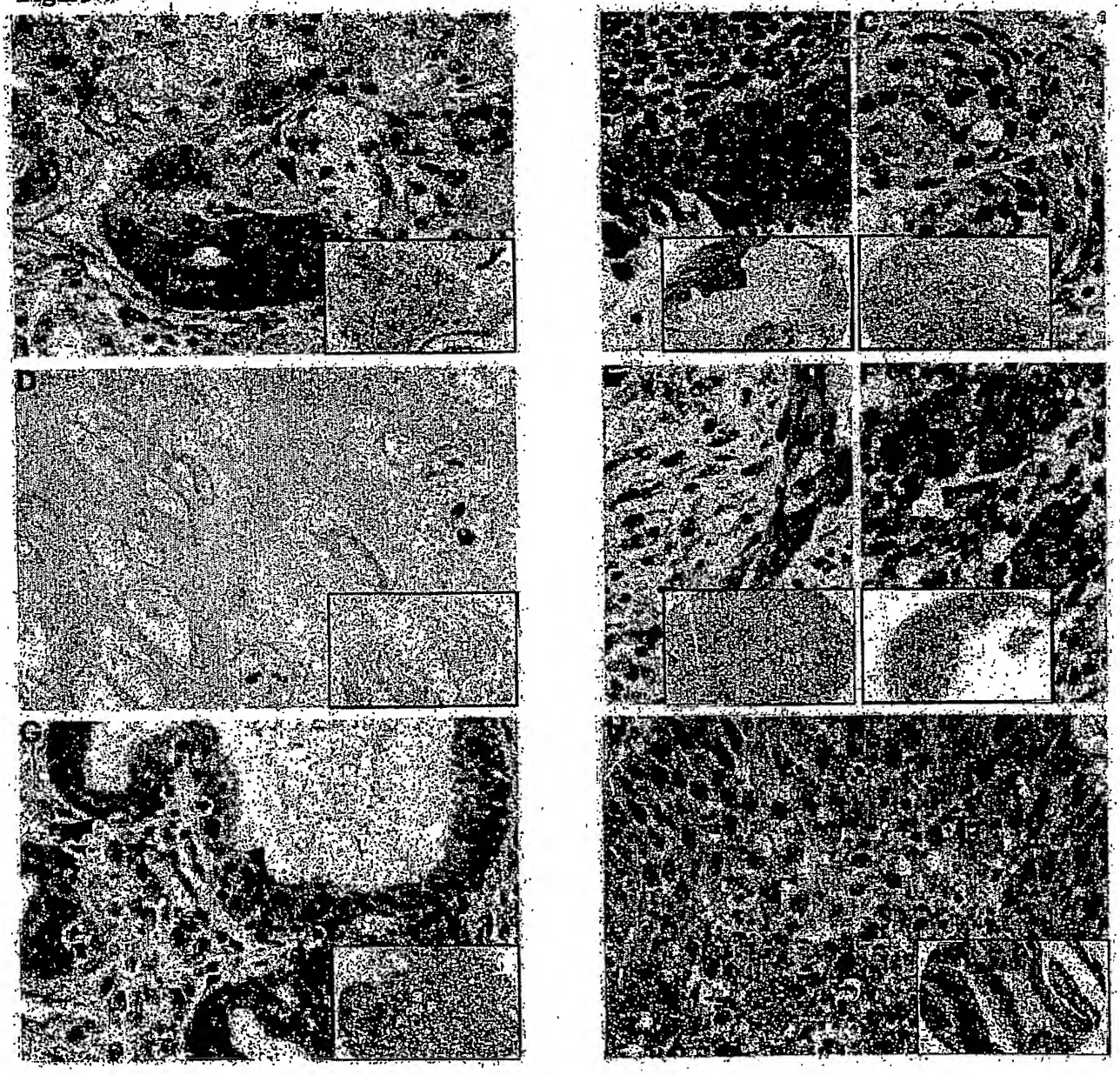

Figure 5

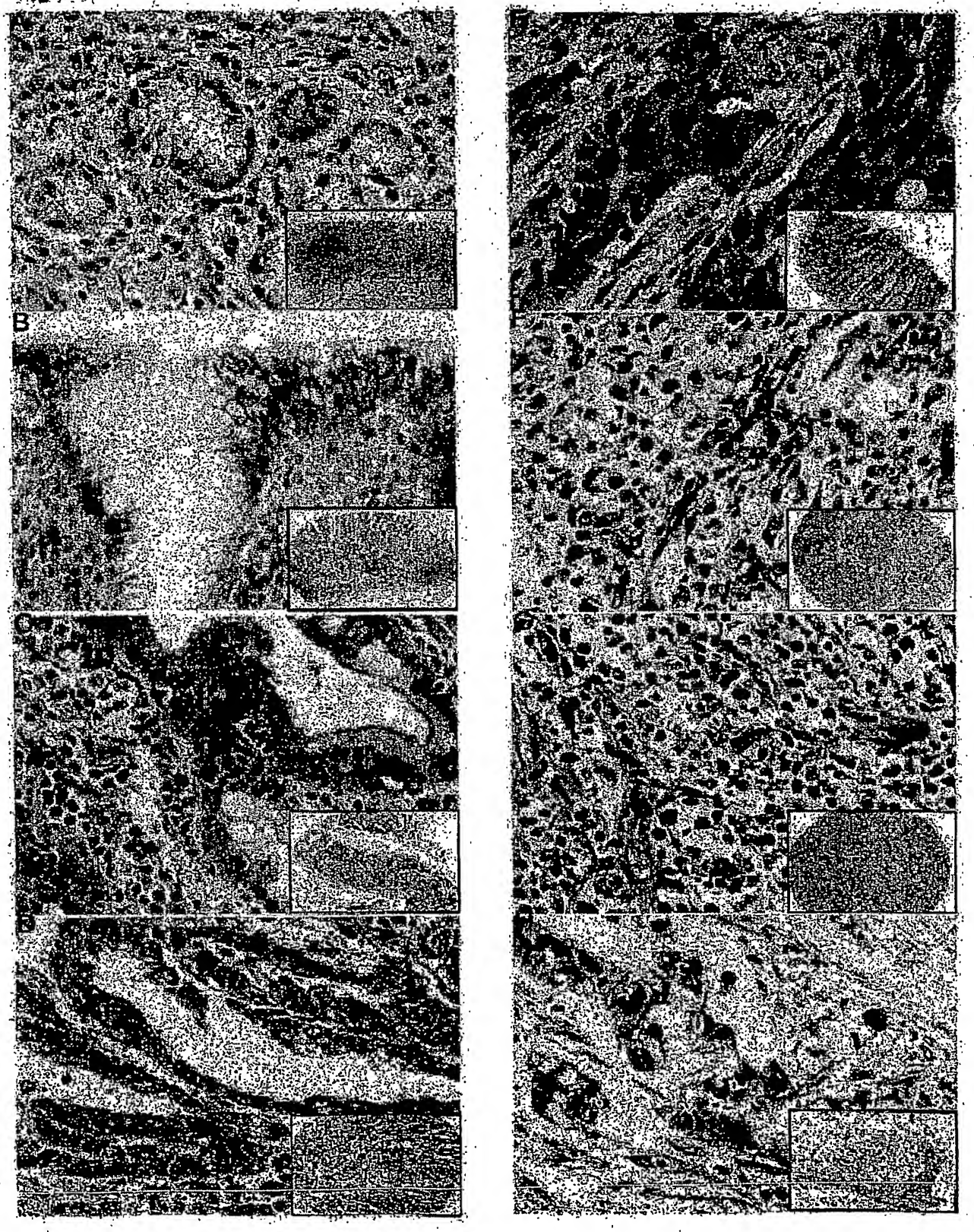

Figure 6

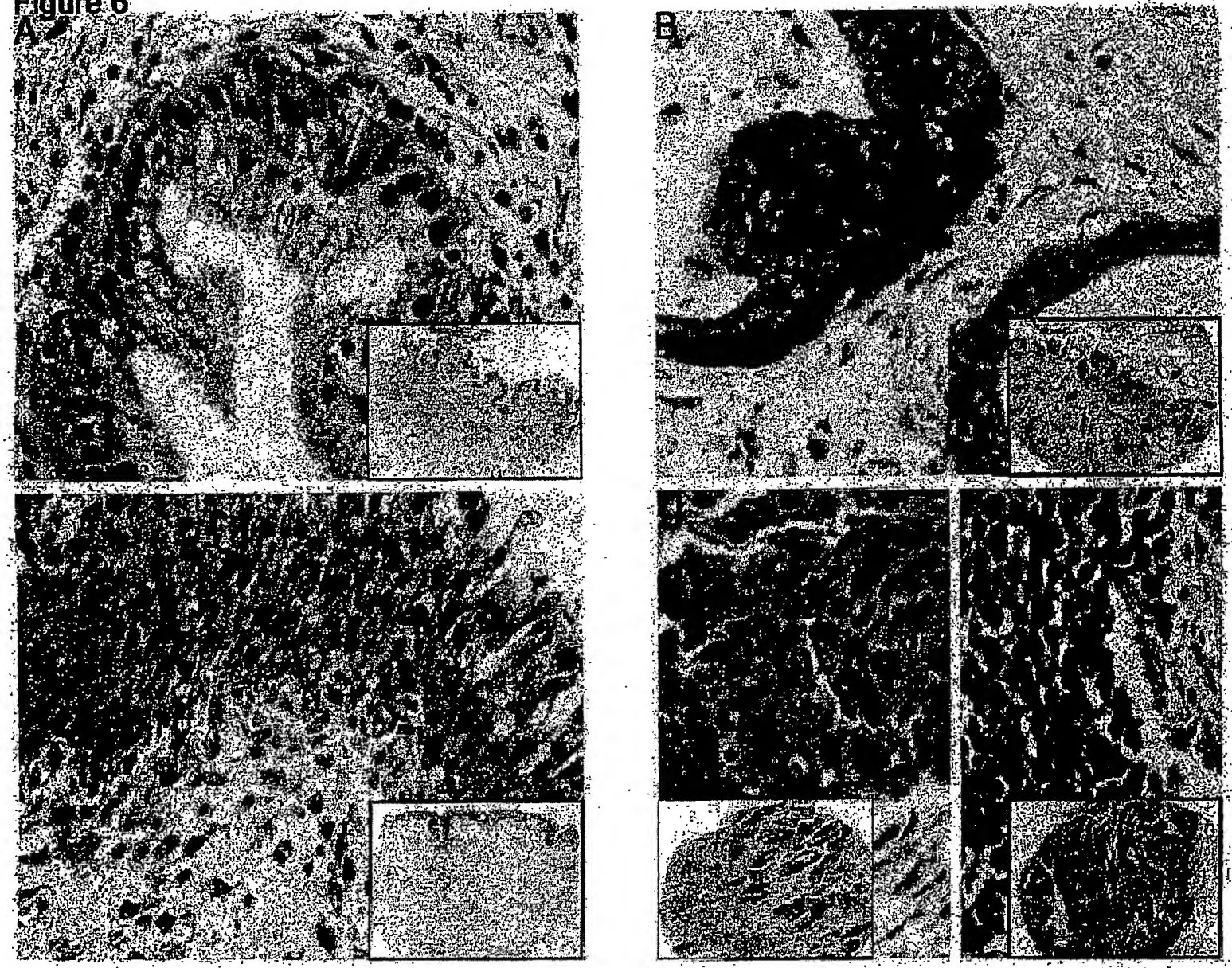

Figure 7

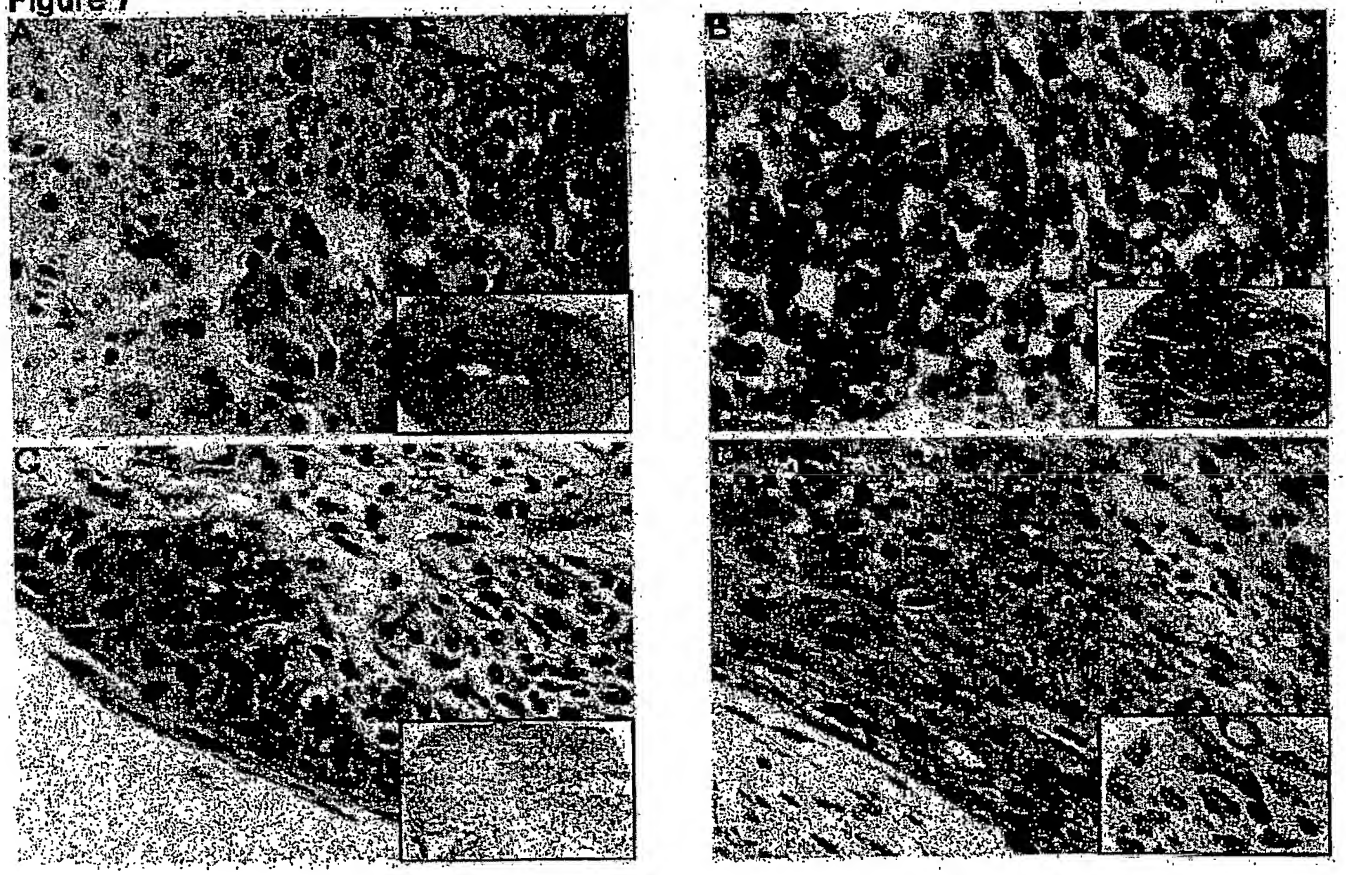

Figure 8

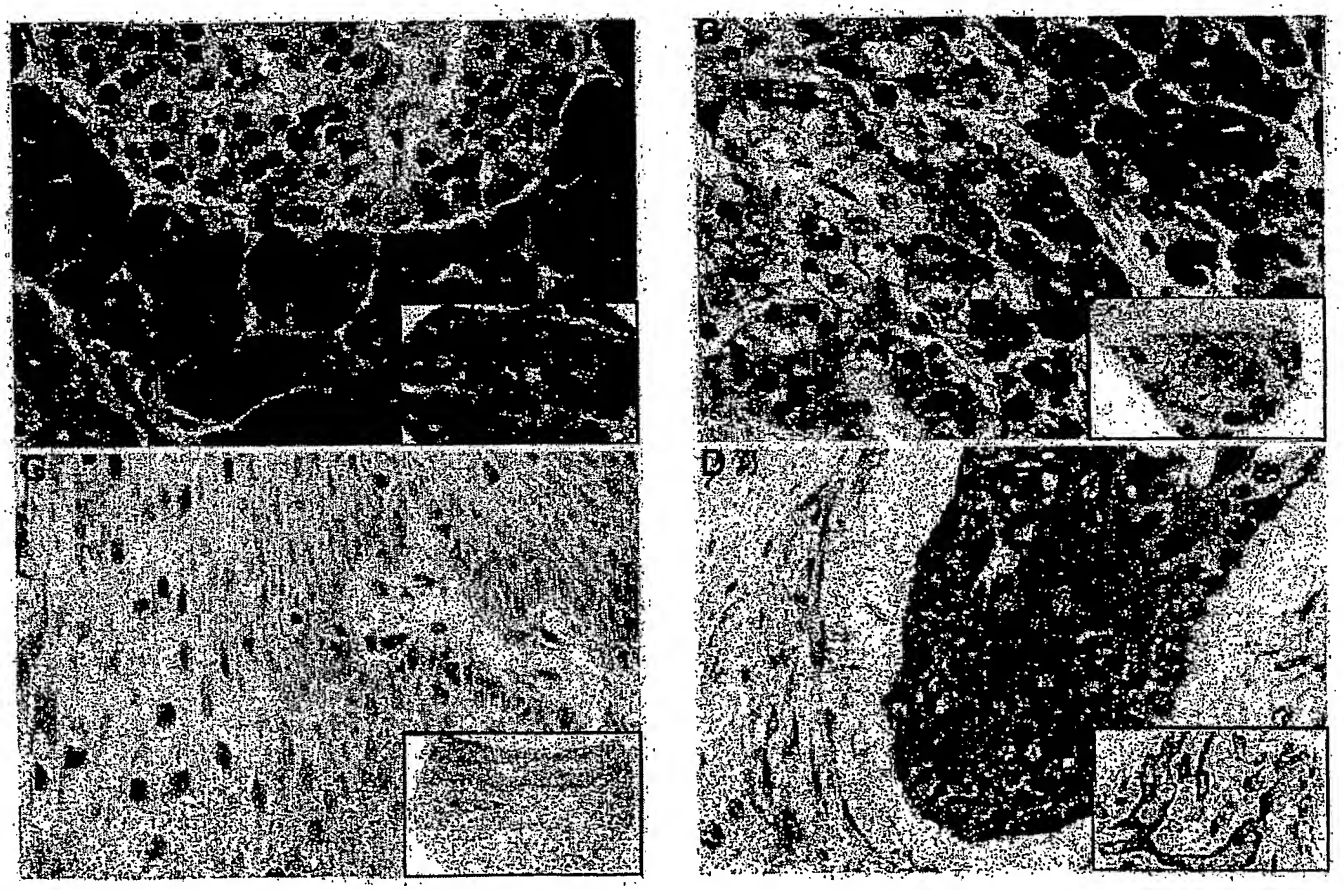

Figure 9

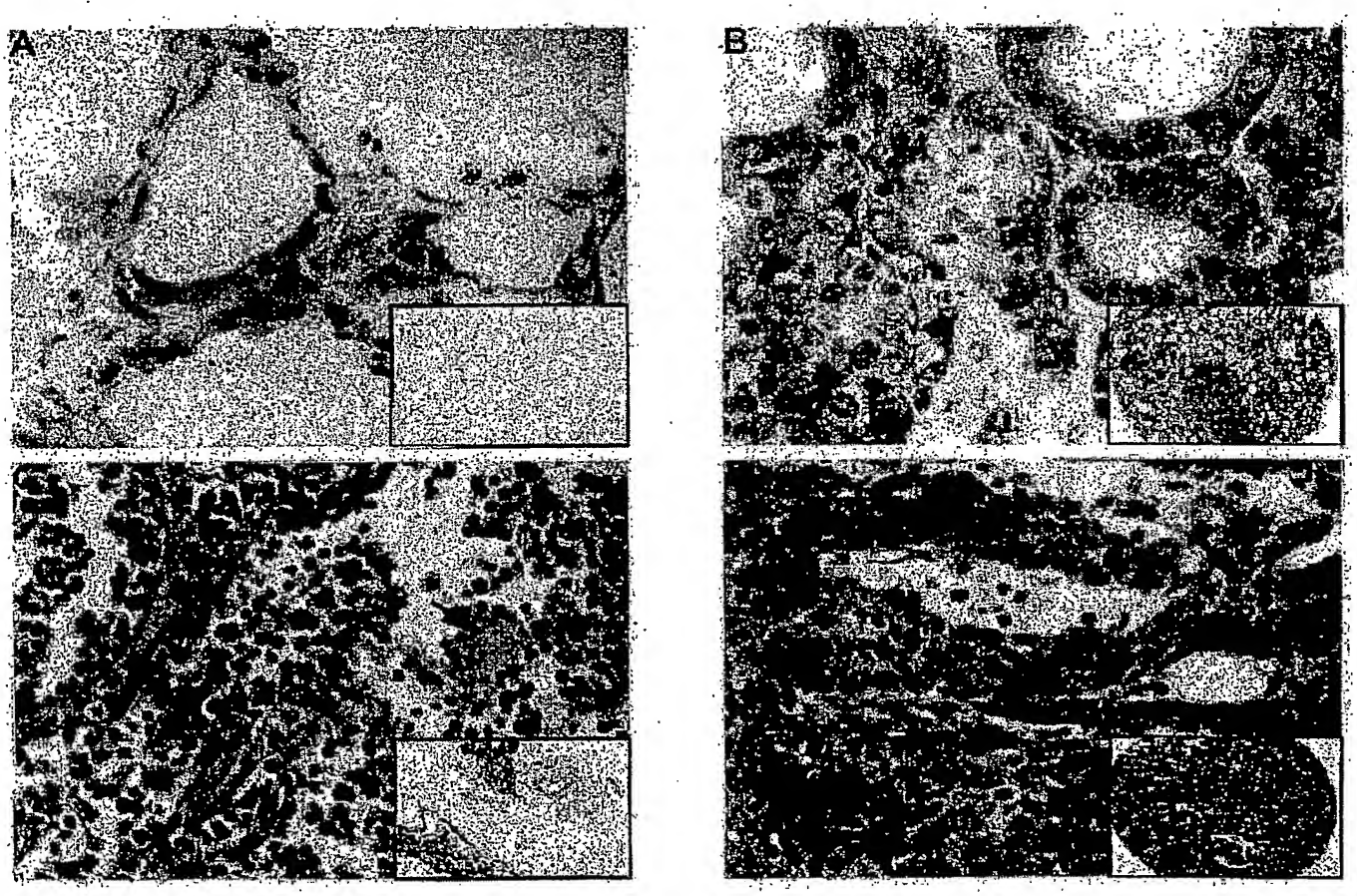

Figure 10

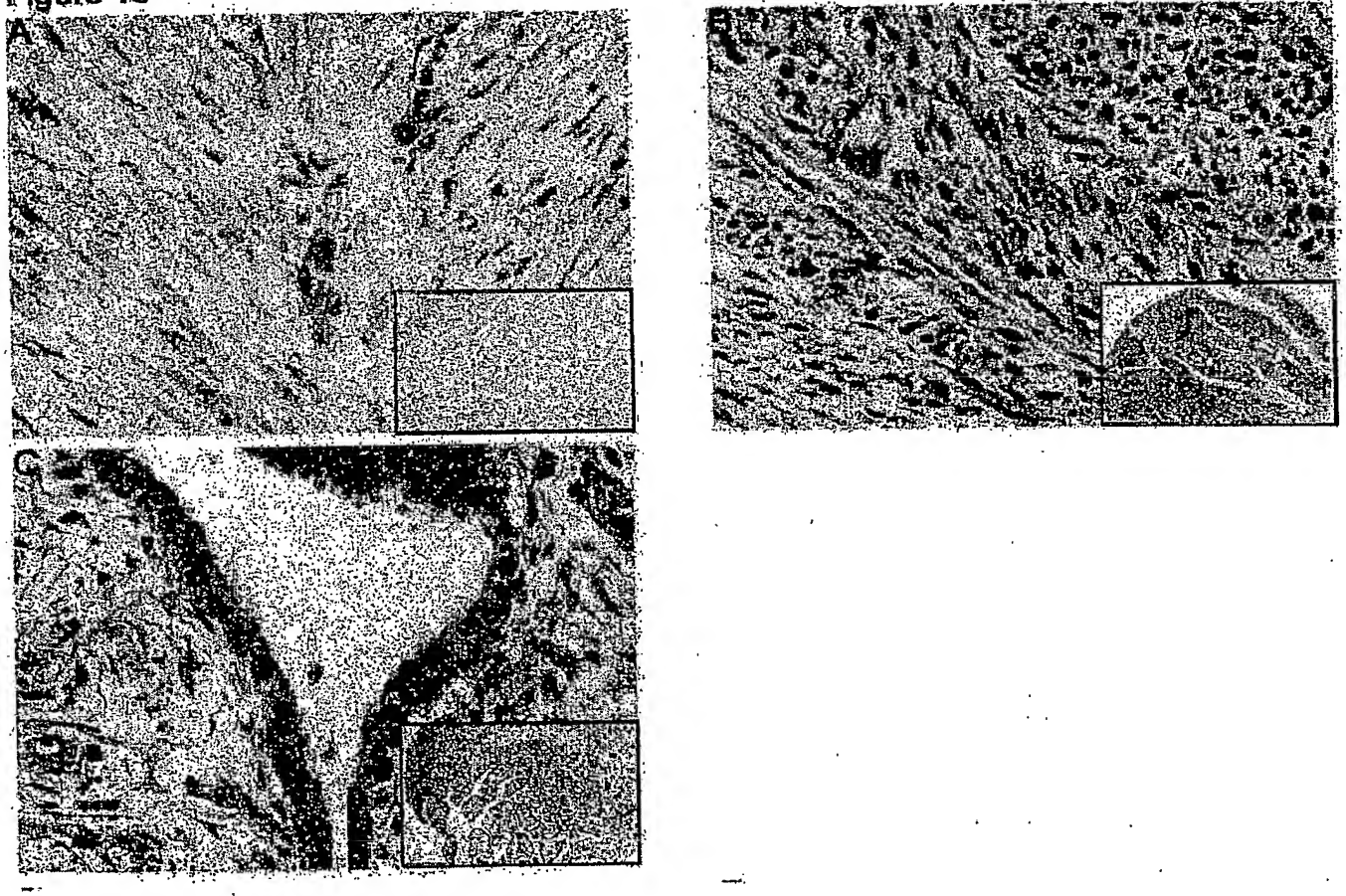

Figure 11

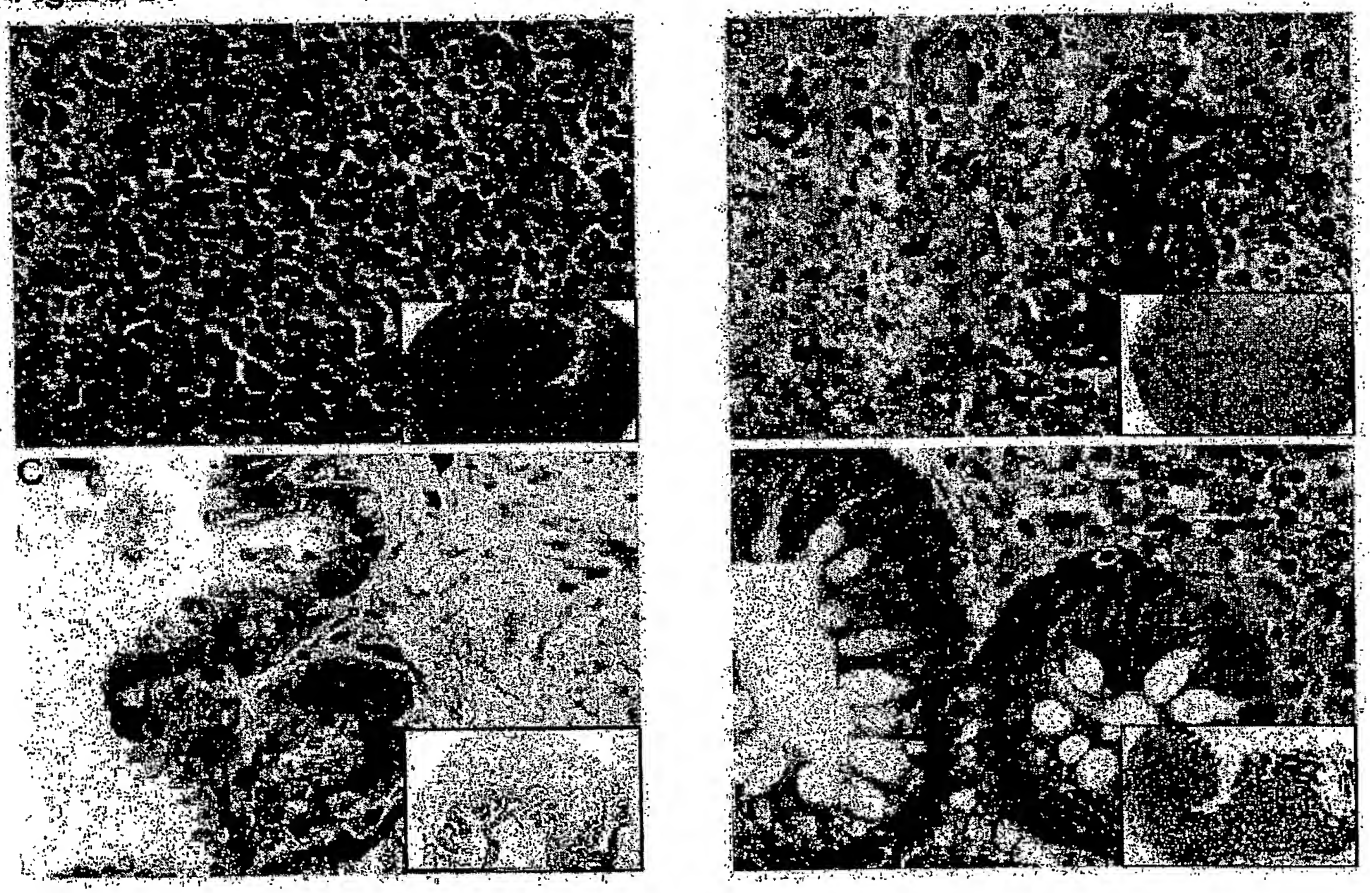

Figure 12

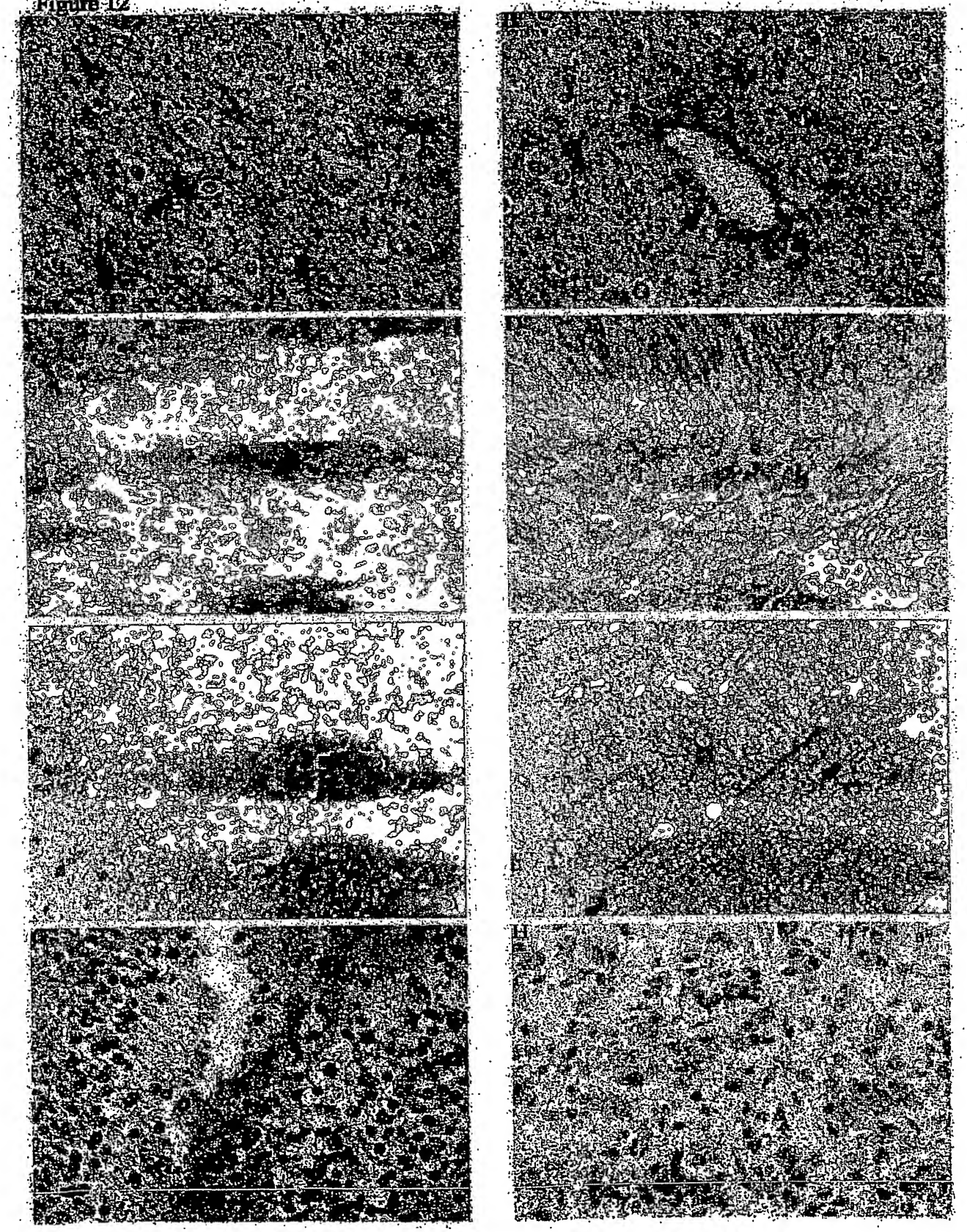

Figure 13

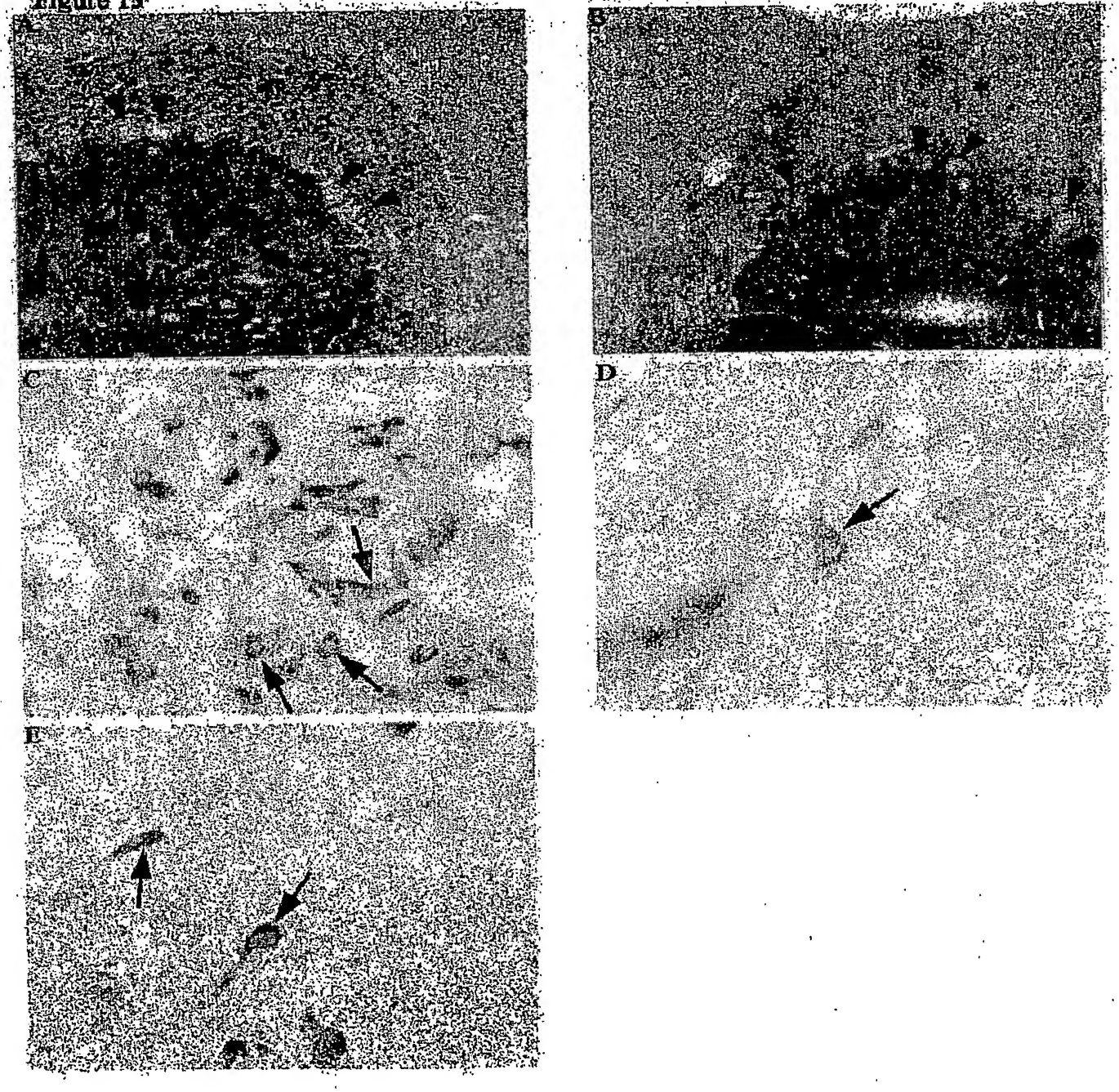

Figure 14